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BARCELONA

Human pluripotent stem cells: towards the definition of new engineering approaches to target heart and kidney disease

Maria Gallo

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Human pluripotent stem cells: towards the definition of new engineering approaches to target heart and kidney disease



Maria Gallo



UNIVERSITAT^{DE}
BARCELONA



Human pluripotent stem cells: towards the definition of new engineering approaches to target heart and kidney disease

Memòria de tesi doctoral presentada per Maria Gallo per
optar al títol de doctora per la Universitat de Barcelona.

Programa de Doctorat en Biomedicina.

A handwritten signature in black ink, appearing to read "M. Gallo".

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A handwritten signature in blue ink, appearing to read "Nuria".

A handwritten signature in blue ink, appearing to read "Elena".

A handwritten signature in blue ink, appearing to read "Pere".

July 2023

Agli affetti incondizionati,

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ABSTRACT

Human pluripotent stem cells (hPSCs) offer a significant advantage in recapitulating key features related to tissue differentiation, morphogenesis and, conversely, human diseases. Taking advantage of their inherent ability to differentiate into the three germ layers, and their unlimited self-renewal capacity, the field has been able to establish novel methodologies to generate three dimensional (3D) self-organized organ-like structures, known as organoids. Moreover, the discovery and development of CRISPR/Cas9 technology has revolutionized our ability to introduce permanent or transient changes in the genome of living organisms and cells. In this context, the host laboratory has previously developed a cellular platform, called iCRISPR2 (iC2), which utilizes an inducible Cas9 under the endogenous TET/ON promoter to enable the efficient genome editing of hPSCs. The iC2 system allows the generation of reporter, knock-out and knock-in hPSC lines. In the present thesis, we have established new tools and methodologies to generate hPSC-derived cardiac models both in two-dimensional (2D) and three-dimensional (3D) culture conditions. To this aim, by exploiting the iC2 platform, we have first generated and characterized hPSC cardiac reporter lines mirroring the endogenous expression of MYH6, MYL2, and SIRPA. Following a well-established cardiac differentiation procedure developed previously in the host laboratory, we have demonstrated the efficient generation of beating cardiac-like cells from these reporter lines in 2D culture conditions. Leveraging this new tool, we have defined a novel approach to generate 3D cardiac-like organoids in a robust and efficient manner that exhibit relevant cardiac differentiation and functional characteristics. Building on the newly defined cardiac-like organoids, we have directed our attention toward establishing a new cardiac *in vitro* model that can emulate important aspects of diabetic cardiomyopathy. For this purpose, we have investigated the impact of exposing hPSC-derived cardiac-like organoids to a diabetic-like insult that was previously reported by the host laboratory to induce early hallmarks of diabetic-like disease in hPSC-derived kidney organoids. Cardiac-like organoids exposed to diabetic-like culture conditions compared to normoglycemia were interrogated at the morphological, transcriptional, and functional levels to assess on the potential phenotypic alterations associated to early hallmarks of diabetic cardiopathy. Furthermore, in the last part of the present thesis, we have focused our

efforts on combining hPSC-derived organoid technology with bioengineering to develop new approaches to enhance the differentiation and functional maturation of hPSC-derived organoids. To this end, we have established novel procedures based on organ decellularization technology to fabricate biomaterials mimicking the biochemical composition of the native organ. We have derived biomimetic hydrogels from decellularized extracellular matrices (dECM) obtained from kidney and heart that have been applied as new cell culture biomaterials during the differentiation of hPSC-derived kidney and cardiac organoid models. All in all, by harnessing the power of organoid technology and pioneering the development of novel biomaterials, we have successfully established advanced hPSC-derived biomodels serving as valuable tools for conducting promising investigations for future advancements in regenerative medicine applications. In view of the current advances in the field of hPSC-derived organoids, genome engineering and bioengineering, in the present thesis, we further discuss the major achievements and ongoing challenges in the development of cardiac and kidney organoids as human *in vitro* models to study organ development and disease.

RESUMEN

Las células madre pluripotentes humanas (hPSC) ofrecen una ventaja significativa al recapitular características clave relacionadas con la diferenciación de tejidos, la morfogénesis y las enfermedades humanas. Aprovechando su capacidad intrínseca de diferenciarse en las tres capas germinales y su capacidad de autorrenovación ilimitada, el campo ha logrado establecer nuevas metodologías para generar estructuras tridimensionales (3D) auto-organizadas similares a órganos, conocidas como organoides. Además, el descubrimiento y desarrollo de la tecnología CRISPR/Cas9 ha revolucionado nuestra capacidad para introducir cambios permanentes o transitorios en el genoma de organismos y células vivas. En este contexto, el laboratorio anfitrión ha desarrollado previamente una plataforma celular llamada iCRISPR2 (iC2), que utiliza un Cas9 inducible bajo el promotor endógeno TET/ON para permitir la edición eficiente del genoma de las hPSC. El sistema iC2 permite generar líneas hPSC reporteras, knock-out y knock-in. En la presente tesis, hemos establecido nuevas herramientas y metodologías para generar modelos cardíacos derivados de hPSC tanto en condiciones de cultivo bidimensionales (2D) como tridimensionales (3D). Con este fin, utilizando la plataforma iC2, primero hemos generado y caracterizado líneas de células cardíacas hPSC que reflejan la expresión endógena de MYH6, MYL2 y SIRPA. Siguiendo un procedimiento bien establecido de diferenciación cardíaca desarrollado previamente en el laboratorio anfitrión, hemos demostrado la generación eficiente de células similares a cardiomiocitos en condiciones de cultivo 2D a partir de estas líneas reporteras. Aprovechando esta nueva herramienta, hemos definido un enfoque novedoso para generar organoides similares al corazón en 3D de manera robusta y eficiente, que exhiben características relevantes de diferenciación y funcionalidad cardíacas. Basándonos en los organoides cardíacos recién definidos, hemos dirigido nuestra atención hacia el establecimiento de un nuevo modelo *in vitro* para el corazón que pueda emular aspectos importantes de la cardiomiopatía diabética. Con este propósito, hemos investigado el impacto de exponer los organoides cardíacos derivados de hPSC a una agresión similar a la diabética, que previamente había sido reportada por el laboratorio anfitrión por inducir marcadores tempranos de enfermedad similar a la diabética en

organoides renales derivados de hPSC. Los organoides cardiacos expuestos a condiciones de cultivo similares a la diabética, en comparación con la normoglucemia, fueron analizados en cuanto a alteraciones morfológicas, transcripcionales y funcionales para evaluar los posibles cambios fenotípicos asociados con marcadores tempranos de cardiopatía diabética. Además, en la última parte de la presente tesis, hemos centrado nuestros esfuerzos en combinar la tecnología de organoides derivados de hPSC con la bioingeniería para desarrollar nuevos enfoques para mejorar la diferenciación y maduración funcional de los organoides derivados de hPSC. Con este fin, hemos establecido procedimientos innovadores basados en la tecnología de descelularización de órganos para fabricar biomateriales que imiten la composición bioquímica del órgano nativo. Hemos derivado hidrogeles biomiméticos a partir de matrices extracelulares descelularizadas (dECM) obtenidas del riñón y el corazón, que se han aplicado como nuevos biomateriales para el cultivo celular durante la diferenciación de los modelos de organoides cardiacos y renales derivados de hPSC. En resumen, aprovechando el poder de la tecnología de organoides y liderando el desarrollo de nuevos biomateriales, hemos establecido con éxito biomodelos avanzados derivados de hPSC que sirven como herramientas valiosas para llevar a cabo investigaciones prometedoras para futuros avances en aplicaciones de medicina regenerativa. A la luz de los avances actuales en el campo de los organoides derivados de hPSC, la ingeniería del genoma y la bioingeniería, en la presente tesis, también discutimos los principales logros y desafíos en curso en el desarrollo de organoides cardiacos y renales como modelos humanos *in vitro* para estudiar el desarrollo y las enfermedades de órganos.

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INTRODUCTION

1. INTRODUCTION

1.1. Human Pluripotent Stem Cells (hPSCs)

The advent of human pluripotent stem cells (hPSCs) has provided the opportunity to derive human tissue-specific cell types *in vitro* and recreate the developmental stages occurring during embryonic organogenesis. Both, human embryonic stem cells (hESCs) (1) and human induced pluripotent stem cells (hiPSCs) (2), the so called hPSCs, are 2 sides of the same coin. They have been a topic of intense research over the past 2 decades due to their remarkable self-renewal property and capability to spontaneously differentiate into cells of the 3 germ layers of the embryo (3,4) (pluripotency) (Fig.1). Indeed, the origin of the term “pluripotency” and its association to stem cells goes back to 1960, when Stevens discovered the formation of a teratoma after the injection of embryonic stem cells in a mouse (5). At the same time, the word “teratoma” has a Greek origin and means “monster”. In histology this term has been coined to refer to tumors containing a mixture of all 3 embryonic germ layers cell types.

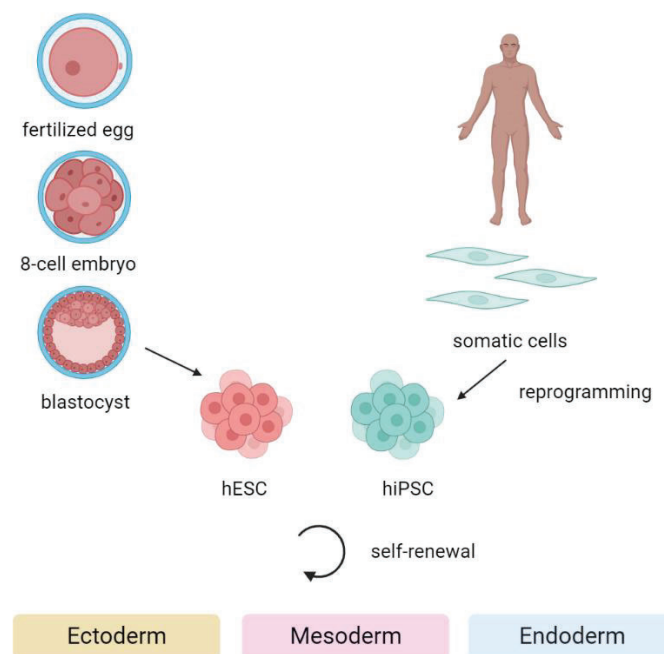


Fig. 1. Human pluripotent stem cells. On the left, a fertilized egg gives rise to an 8-cell embryo, which forms a blastocyst. hESCs can be isolated from the inner cell mass and cultured *in vitro*. On the right, hiPSCs are derived by reprogramming of somatic cells into a pluripotent state using a specific cocktail of transcription factors. Both hESC and hiPSC, the so-called hPSCs, have self-renewal and pluripotency properties [Figure created with biorender.com].

1.1.1. hPSCs in the embryo: from biology to cell culture

During embryonic development in mammals, the blastocyst is formed of an inner cell layer (inner cell mass- ICM) and an outer cell layer (trophectoderm-TE). Advances in the field of tissue culture, allowed the isolation of mouse ESCs (mESCs) from the ICM of a preimplantation blastocyst by 1981, recognizing the impact of this discovery with the Nobel Prize for Medicine in 2007 (3,6) of Martin Evans. During the 1980s different laboratories described techniques and approaches to propagate and differentiate mESCs leading to the possibility to further isolate ESC from non-humans primates (7) and, after 3 years, from human preimplantation blastocysts (1). Nowadays, around 500 hESC lines are registered to the National Institutes of Health Human Embryonic Stem Cell Registration (http://grants.nih.gov/stem_cells/registry/current.htm). The majority of hESCs have been derived from embryos donated to biomedical research (8). Immunosurgery, mechanical ICM dissection (MID), and whole embryo culture (WEC) are the techniques developed to isolate hESCs. At the beginning, two important challenges confined their application: ethically, hESCs have always been target of discussion because of the destruction of a “life”, and immunologically, because of the rejection of cells differentiated from hESCs upon allogenic cell transplantation (9). To keep under control these issues, in 2002 was founded the International Society for Stem Cell Research (ISSCR). The ISSCR is a professional organization whose objective is the promotion of stem cell research and its application for treating human diseases. The society is composed of scientists, clinicians, ethicists, and other professionals from around the world working on stem cell research. The ISSCR provides a forum to share knowledge and establish best practices for the stem cell field. In this regard, the ISSCR publishes guidelines for stem cell research and clinical translation. The latest updated guideline was published in 2021. This new guideline includes some revisions related to the application of genome editing, as well as an increase in examples of appropriate and inappropriate clinical applications. Main advances involve culture of human embryos and stem cell-derived models of embryo development (embryo-like entities and organoids), chimeras, *in vitro* gametogenesis from cells, mitochondrial replacement techniques, somatic and germline genome editing, enhanced guidance for procurement of stem cell lines, and more robust clinical translation guidance. Overall, the new guideline emphasizes the importance of

inclusion of embryo research, especially as hESCs or hiPSCs can provide a preliminary model for testing methodology before moving onto animal models. (10).

1.1.2. Human induced pluripotent stem cells: from early generation to latest advances in somatic cell reprogramming

In 2007, Takahashi and Yamanaka discovered that somatic cells can be converted artificially into cells resembling pluripotent stem cells, the so-called iPSCs, which culminated with the prestigious recognition of the Nobel Prize in Medicine and Physiology by 2012. In their seminal work, the authors ectopically expressed the so-called “Yamanaka cocktail”, constituted of Oct4, Sox2, Klf4 and cMyc (OSKM) (2,11). OSKM factors were the evidence that science is not easy and the proof of an achieved milestone for Yamanaka. Indeed, the discovery of this “magical cocktail” is the result of a screening process that started almost 5 years before, meticulously exploring all the possible combinations for the overexpression of 24 transcription factors involved in several cellular processes, including pluripotency, cell survival and differentiation.

The use of hiPSCs not only addresses ethical concerns related to the use of hESCs, but also enables the generation of patient- specific pluripotent stem cells with specific mutations, which is essential for disease modeling. In the last 15 years, several approaches have been adopted to reprogram somatic cells. These methods can be divided into integrative and non- integrative. Integrative methods refer to foreign DNA sequences encoding reprogramming factors that are inserted into the genome, while non-integrative do not require permanent genetic modification (12,13). Obviously, safety and efficiency is inversely proportional for the 2 approaches, the non- integrative are safer and less efficient than the integrative and *vice versa* (14).

Both hESCs and hiPSCs express transcription factors of pluripotency, such as OCT4, NANOG, SOX2, as well as surface markers including tumor rejection antigens Tra-1-81 and tra-1-60. Moreover, they are positive for stage-specific-embryonic antigens (SSEA3 and SSEA4). hiPSCs, together with hESCs, offer immense potential for biological studies and applications in the field of regenerative medicine in which they have become the holy grail as a therapeutic tool for treating a wide range of life-threatening diseases. The main diseases in which hiPSC-derived cells have showed promising results in first phases of clinical trial are above all neurodegenerative

diseases, cardiac diseases or, due to the pandemic, for COVID-treatments. One of the first trials relayed on the generation of dopaminergic neurons derived from hiPSCs showing improved function in a primate model for Parkinson's disease (15). Other clinical trials for heart failure test the hypothesis that cardiomyocyte-derived hiPSCs can be implanted via engineered heart muscle leading to sustainable remuscularization in the failing heart (16). In the last years, given the urgency of COVID-19, another clinical trial has developed hiPSC-derived NK cells with the objective of identifying the maximum tolerated dose of a drug for the treatment of this respiratory virus (17). Despite the promises and opportunities addressing the efficacy of hiPSCs to restore organ function since 2006, clinical trials proving the efficacy of these cell products are still scarce.

1.2. Model systems to study tissue and organ development from hPSCs

Historically, the development of therapeutic treatments has relied on classical cell lines and animal models. Typically, disease mechanisms are first investigated in invertebrates and then analyzed for evolutionary conservation in mammals. However, the step prior clinical applications remain the bottleneck in biology due to the over 75 million years of divergent evolution between rodents and humans. This deep interspecies distance makes difficult to faithfully recapitulate human pathologies (18). For this reason, hPSCs are being identified as an appealing platform for overcoming the above-mentioned limitations.

1.2.1. *In vitro* cell culture in 2D: how to capture early stages of cell lineage specification and differentiation

In vitro cell culture is a valuable tool for studying cell behavior and function, particularly during early stages of lineage specification and differentiation. Two-dimension (2D) cell culture has been the first and most used method for the study of tissue and organs development. Exploiting hPSCs, worldwide researchers have generated procedures enabling the production of different cell types mirroring tissue-like cells. In this manner, it has been possible to start addressing complex questions in the Petri dish, including both early processes guiding tissue specification and formation as well as disease

origin and progress. Despite the enormous impact of those early studies and the amenability of these culture formats for High-throughput screening (HTS), many challenges still remain. First, 2D culture does not provide organ-like constraints which are well-known to guide tissue formation and function (including both physical and chemical cues), at the same time, 2D culture conditions preclude the study of cell-to-cell interactions in similar fashion as accounts in the tissue, in which multiple cell types are arranged in the three-dimensional (3D) space. Another important aspect in the hPSC field is that the lack of a 3D-like environment impacts on cell differentiation extent. Therefore, in 2D the maturation of hPSCs in culture is limited. Hence, the 2D scenario mimics embryonic stages of differentiation (19). In this regard, the field is under a present open debate in which 2D cell culture conditions are envisioned as the minimal culture set up to model early-age onset disease rather than conditions compromising adult tissues.

1.2.2. *In vitro* cell culture in 3D to capture early stages of cell lineage specification and differentiation: from embryoid bodies to organoids

Both two-dimensional (2D) and three-dimensional (3D) *in vitro* cell culture systems represent a powerful tool in the field of cell biology. Traditional 2D cell culture methods, however, do not fully recapitulate the complex and dynamic nature of cells in their native three-dimensional environment. Recent advances in 3D cell culture techniques have enabled researchers to better mimic the *in vivo* microenvironment of cells, resulting in improved cell viability, proliferation, and differentiation. In this regard, the so-called organoids have been envisioned as an affordable solution overcoming the limitations of 2D cell culture. Compared to the 2D system, they have been shown to recapitulate the complexity of the architecture and cell-cell interactions of native organs. The 3D culture system can be defined as suspension culture which avoid direct physical contact to the plastic dish. This can be accomplished through either scaffold or scaffold-free techniques. In this regard, the roots of 3D culture system field can be traced back to the early 20th century with a scaffold-free technique when Harrison used for the first time the hanging drop cell culture method to culture neural explants (20). In 1907, Wilson made a significant breakthrough by describing the initial *in vitro* attempt at organism regeneration. He demonstrated that dissociated sponge

cells could self-organize and regenerate an entire organism (21). Several decades later, various research groups conducted dissociation-reaggregation experiments to generate diverse types of organs from dissociated amphibian pronephros (22) and chick embryos (23). Successively, the discovery of scaffolds, biological or synthetic hydrogels, emerged as a crucial milestone in the quest to mimic the natural microenvironment of an organ. In 1956 a significant step was taken with the generation of collagen hydrogel reconstituted from rat tails (24), and 20 years after, the first isolation of Matrigel from matrix of chondrosarcomas occurred (25).

The proper shift from 2D to 3D organoid research began when Eiraku et al. (26) were able to generate cerebral cortex tissue from ESCs using the 3D aggregation culture method. In 2009, another landmark study from Sato et al. (27) demonstrated that single leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5)-expressing adult intestinal stem cells could form 3D intestinal organoids in Matrigel. These organoids exhibited self-organization and differentiation into crypt-villus structures independently of a mesenchymal niche. This pioneering work was the first to establish 3D organoid cultures derived from a single adult stem cell (ASC), laying the foundation for numerous subsequent breakthroughs in the organoid field.

Historically, the word “organoid” was used to refer to intracellular structure organelles by the 1950s and 1960s (28,29) as well as to refer to tumours (30) or abnormal cellular growths (31). More importantly, other works refer to the term organoid to name cellular clusters that maintain the structural characteristics of the tissue of origin (32). From 1980, works describing the generation or use of 3D cultures included the use of the word organoids. In the following years the meaning of the term was changed and associated to “a unit of function of a given organ that is able to reproduce, in culture, a biological structure similar in architecture and function to its counterpart *in vivo*” (33).

In the field of hPSCs, organoids represent a methodological evolution of the embryoid body (EB) culture method described in 1995 (34). EBs can be generated by the aggregation of hPSCs in 3D culture conditions and have been extensively shown to simulate the gastrulation of developing embryo and generate the 3 germ layers. Early studies exploiting mESCs set the bases for the use of this approach to study later on hPSC differentiation *in vitro*, including processes related to early embryonic development and later stages of differentiation. During the last decade, most of the

protocols aiming to generate organoids from hPSCs take advantage of early phases relaying on the generation of EBs which are further maintained in 3D culture conditions in the presence of morphogens and soluble factors known to induce early stages of organogenesis (26,35–37). Nowadays, many protocols have been generated for almost any organ (i.e. brain, lung, kidney, heart, etc.) (38–41).

In the following sections, this introduction will focus on the revision of early and current works describing on the generation of heart and kidney organoids, the 2 models used during this thesis.

1.2.3. The role of the extracellular matrix in hPSCs differentiation: impact in kidney and heart tissues

The extracellular matrix (ECM) is a complex network composed of different proteins, such as collagens, proteoglycans, glycoproteins, and glycosaminoglycans, which surrounds and supports cells in multicellular organisms. Its structure is dynamic and varies in composition depending on the specific tissue and organ. ECM interactions play a crucial role in cell growth, organ development and tissue regeneration because of its structural and anchorage role. In recent years, ECM has been also studied for its functional properties, in particular its capability of producing signals influencing cell fate and differentiation (42). Cells isolated from their native matrix may diminish specific characteristics in the absence of a supportive microenvironment. Therefore, it is crucial to provide tissue-specific ECM substratum *in vitro* for cultivating cells to sustain their particular features (i.e., coating, feeder layer, etc) (43). In this regard, decellularized ECM (dECM) substrates can closely mimic cellular microenvironments of native tissues and organs. The decellularization process consists in the removal of the cellular content from a tissue or organ while preserving the native ECM composition and architecture integrity. The first decellularization occurred in 1948, when acellular homogenates were prepared by pulverizing muscle tissue at very low temperatures to remove cellular components (44). Over the years, decellularization techniques have been improved and currently, several decellularized tissues, such as heart, lung, liver, kidney and pancreas, are commercially available for tissue repair and regeneration in clinical applications (45). Nowadays organ decellularization can be achieved through perfusion of decellularization agents through the organ

vasculature or by immersion/agitation of tissue pieces in decellularization agents (46,47). In the last decade, dECM from organs and tissues have been envisioned as promising organ-specific scaffolds for tissue engineering applications. Bioengineering approaches offer viable solutions to address significant challenges related to hPSCs differentiation, such as maturation and functionality. The use of biomaterials derived from different organs combined with hPSCs has enabled to improve differentiation protocols, and even the partially or totally reconstruct of mouse (27) and human whole organs (48,49). In particular, several findings have showed the promising application of biomaterials derived from kidney and heart tissues in combination with hPSCs. Ross et al. demonstrated that when seeding mESCs into a decellularized rat kidney, primitive precursor cells spontaneously populated and proliferated within the glomerular, vascular, and tubular structures, undergoing a transition from their embryonic properties (50). Also, Bonandrini et al. showed the loss of pluripotency and the start of differentiation toward meso-endodermal lineage when mESCs were perfused into an entire decellularized rat kidney (51). More recently, porcine renal dECM-derived hydrogel was used to culture hPSC-derived kidney organoids. Single-cell transcriptomic analysis for this study revealed that in the presence of dECM, kidney organoids developed more mature patterns of glomerular development and higher similarity to human kidney than those cultured without kidney dECM (52).

Additionally, studies performed on cardiac tissues showed that the heart dECM can drive differentiation and maturation of cells into a cardiac cell fate. In 2010, DeQuach et al. fabricated naturally ECM-derived coatings from decellularized heart and skeletal muscle derived from the porcine intercostal muscles. This coating enabled the maturation of hESC-derived cardiomyocytes (53). Successively, French et al. demonstrated that a coating of porcine cardiac-derived dECM enhanced the adhesion, maturation, proliferation, and survival of cardiac progenitor cells compared with collagen alone (54). Studies were performed also in 3D, when Duan et al. prepared different ratios of porcine cardiac dECM-derived hydrogel. They observed increased cardiac markers and contracting cells in differentiated hESC-derived EBs encapsulated with higher percentage of ECM hydrogels (55). Moreover, the host laboratory demonstrated that hPSC-derived cardiomyocyte-like cells (CLCs) co-cultured on human ventricular dECMs for 10 days displayed better electrophysiological responses compared to Matrigel coating (56). In conclusion, bioengineering

approaches using dECM represent an affordable approach to provide structural and biochemical cues that highly reproduce the *in vivo* tissue milieu potentially impacting cell differentiation and maturation.

1.3. Model systems to study cardiac development and disease

Cardiovascular diseases (CVD) are one of the leading causes of death worldwide (57–59). In the last decades different *in vitro* solutions have been proposed to target CVD. However, the human heart is one of the most complex organs to model *in vitro* (60,61). In this section of the Introduction, models based on the use of hPSCs to understand cardiac development and disease are going to be discussed. First, information on cardiac development based on fundamental knowledge from animal models, as the embryonic mice, are going to be highlighted. Second, we are going to introduce the different cell culture model systems based on the use of hPSCs to model early phases of cardiac development and differentiation as well as to understand CVD.

1.3.1. *In vivo* cardiomyocyte specification and differentiation

The mammalian cardiovascular system is the first system to develop due to the growing embryo's increasing metabolic demand. Interestingly, during development the heart grows faster than the rest of the body. Cardiac development is a complex process involving intricated molecular signaling, which brings to the proper formation of structures and spatial configuration changes in the adequate timing. In this first part, we are going to quickly explain the mammalian cardiac development with the pathways involved in it until arriving at the cardiomyogenesis.

I. Mammalian cardiac development

As already mentioned, the heart is the first functional organ that forms during embryonic development in mammals (62). The formation of the heart begins during gastrulation, at the end of the 2nd week of human embryonic development (63). The human embryo at this stage consists of 3 germ layers: endoderm, mesoderm, and ectoderm. The major contribution to the heart tissue is given by the mesoderm layer. It originates from 2 sets of mesoderm- derived cardiac progenitor cells (CPCs) known as first and second heart field (FHF and SHF) (64,65). In mice, the FHF originates

from cells in the anterior lateral plate mesoderm, which forms a crescent shape at E7.5 (approximately, 2 weeks of gestation in humans). At E8 in mice (approximately, 3 weeks of gestation in humans), cells of the FHF form the primary heart tube (PHT), and cells of SHF migrate into the PHT from both sides of the heart leading to the expansion of the heart. PHT consists of 2 layers separated by ECM for reciprocal signaling: the outer layer of myocardial cells and the inner layer of endocardial cells (66). Afterwards, the looping of the PHT allows the formation of the 4 cardiac chambers (i.e., left and right atria and left and right ventricles) by E9.5 to E14.5 during mice and at week 4 to 8 of human embryo development, respectively. During this stage, a subset of epicardial cells undergoes epithelial-to-mesenchymal transition (EMT) to become epicardial-derived cells (EPDCs), which will give rise to the epicardium, the mesothelial heart's outer surface. Later, the differentiation of the outflow tract (OFT) brings to the formation of a compact myocardium and septum from the outer part of the ventricular myocardium. It is important to mention that in humans, by the end of the 1st semester, the major components of the heart are formed when the division of the OFT generates the aorta and pulmonary artery (Fig. 2).

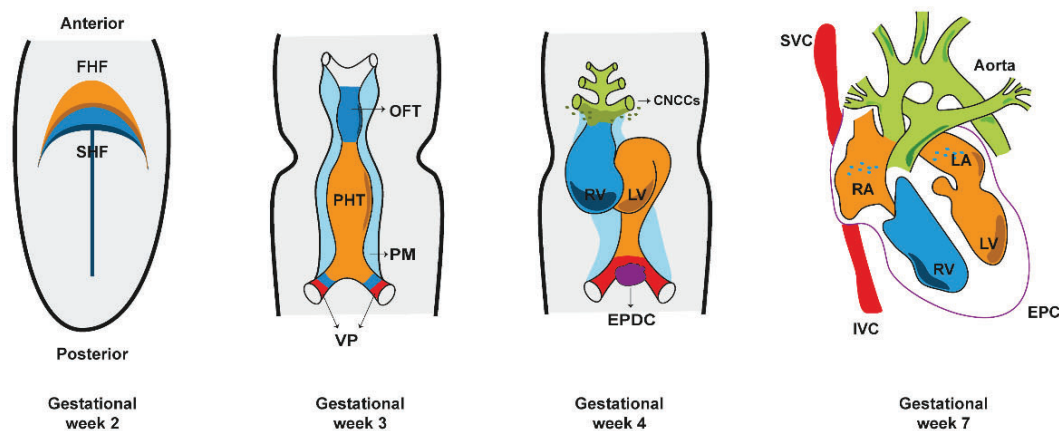


Fig. 2. Development of human heart at embryonic stage. At week 2 of gestation, the mesoderm-derived cardiac progenitor cells migrate toward the anterior side of the embryo to form the first heart field (FHF) and second heart field (SHF). The FHF gives rise to the primary heart tube (PHT) and gradually to the left ventricle (LV), and parts of the right and left atria (RA and LA, respectively). The SHF forms the outflow tract (OFT), then the right ventricle (RV) and later the pulmonary artery and the base of aorta. Instead, cells at the venous pole help the formation of vena cavas (SVC and IVC). By gestational week 4, the cardiac neural crest cells (CNCCs) migrate from the dorsal neural tube, forming smooth muscle cells within the aortic and pulmonary arteries. Epicardium-derived cells (EPDC) give rise to the epicardium (EPC), the mesothelial cell sheet covering the heart's outer surface [Figure adapted from (67)].

II. Signaling pathways during cardiac development

Mammalian cardiac development is the result of an intricate and coordinated interaction among gene expression, signaling pathways, transcriptional regulators, and transcription factors. The first molecular markers for cardiac progenitors are the transcription factors MESP1 (mesoderm posterior 1) and MESP2 (mesoderm posterior 2) (68). MESP1 triggers the expression of some cardiac specification factors including: NKX2.5, Insulin gene enhancer protein 1 (ISL-1), and myocardin (MYOCD). Also other transcription factors control the expression of cardiac genes at early stages of myocardial differentiation, such as GATA binding- protein 4 (GATA4), myocyte enhancer factor 2C (MEF2C), basic helix-loop-helix factors heart and neural crest derivatives expressed 1 (HAND1) and 2 (HAND2) and Brachyury (T)(69). The specification of different populations of cardiac progenitors' cells from the cardiac mesoderm is regulated by the Wnt- β -catenin, Notch and FGF signaling pathways (70–72). Moreover, the interplay between canonical Wnt and BMP signaling gradients are essential for the early steps in cardiac development. At later stages during cardiac development, the constitutive activation of Wnt signaling helps the specification of SHF progenitors and β -catenin assists their expansion; whereas the inactivation of β -catenin results in a partial loss of SHF progenitors (73–77). FGF, BMP, Hedgehog (Hh), Notch and non-canonical Wnt signaling are also involved at various stages during FHF and SHF progenitor specification, expansion, and differentiation (78–82) (Fig. 3).

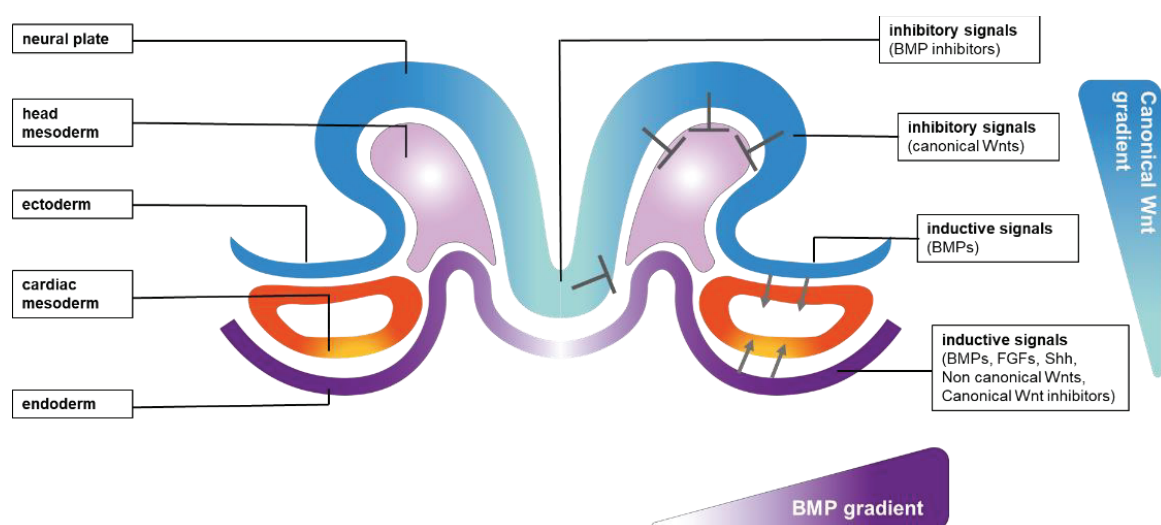


Fig. 3. Schematic representation of a transverse section of an early-stage mammalian embryo showing the reciprocal signals involved in shaping the cardiac precursors *in vivo*. Cardiogenic secreted factors crucial for the formation of the cardiac mesoderm are produced by the anterior endoderm and ectoderm (BMP, FGFs, Shh, Wnts). These signals counterbalance the effects of cardiac inhibitors such as canonical Wnts and BMP inhibitors, which are released by the neural plate. The cardiac mesoderm is characterized by a region exhibiting elevated BMP activity and reduced Wnt activity [Figure adapted from (83)].

III. Cardiomyocyte development (cardiomyogenesis)

Cardiomyogenesis is the transition from fetal to adult cardiomyocytes (CMs) that involves changes into cell structure, metabolism, function, and gene expression. This process requires down-regulation of pro-proliferative signals (Wnt- β -catenin) (73,74,76) and the up-regulation of differentiation pathways (BMP and Sonic Hedgehog) (84). The major hallmarks of cardiomyocyte development can be resumed into the maturation of myofibrils, electrophysiology, and metabolism. Myofibrils are structures that allow the contraction of cardiomyocytes (85,86). They are constituted of repeated subunits better known as sarcomeres. Each sarcomere is built of thin filaments (sarcomeric actin, troponins, tropomyosin), thick filaments (myosin heavy and light chains), Z-lines (actinin), M-lines (myomesin) and TTN (titin) (87) (Fig. 4).

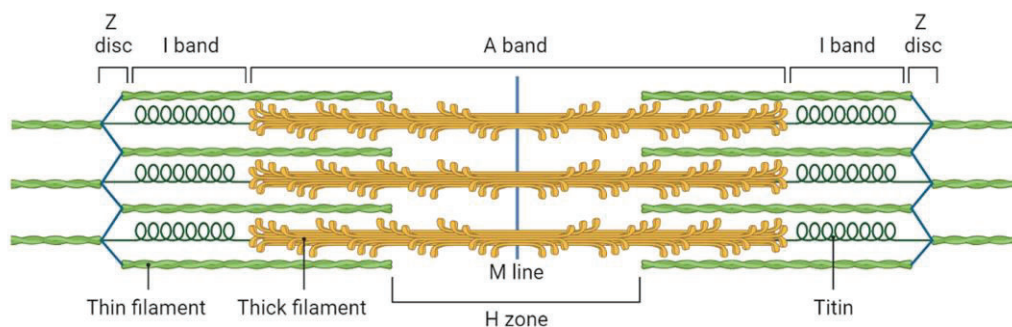


Fig. 4. Schematic representation of a sarcomere. Thick and thin filaments overlap in the A band region, whereas the I band is formed from the thin filament only. Thin filament is composed of tropomyosin and troponin complexes interacting with actin. [Figure created with biorender.com].

The switching of different sarcomeric isoforms, due to transcriptional changes or alternative splicing, is a key element of myofibril maturation. In mouse the switching time from the fetal myosin heavy chain 7 (Myh7) to adult Myh6 isoform is well-known while in humans this process is still not clear. Importantly, the use of hPSCs-derived cardiomyocytes to understand this question indicates that MYH6 expression can be

detected early during cardiac differentiation. Conversely, the detection of MYH7 expression human developing cardiomyocytes *in vivo* (by week 5 of gestation) indicates that this sarcomeric isoform's switch also happens (88–90). Isoform switching also occurs in myosin light chains. MYL7 is highly expressed in early fetal cardiomyocytes, but then shift to MYL2 in ventricular mature cardiomyocyte and remain restricted to atrial cardiomyocytes (91,92). Moreover, slow skeletal troponin I (TNNI1) present in fetal stages, is replaced by cardiac troponin I (TNNI3) (93).

Heart contraction is a mechanism controlled by cardiac ion channels regulating electrical impulses and cytoplasmic calcium concentration. Immature and mature cardiomyocytes differ also under electrophysiological levels. In premature cells, the resting membrane potential is higher than mature cardiomyocytes, because of the action of rectifying potassium channels (Kir) Kir2.1 and Kir2.2 (KCNJ2 and KCNJ12) (94,95). The action potential is quicker in mature cells due to the rapid opening of voltage-gated sodium channels (SCN5A), which permits sodium influx and membrane depolarization (96,97). Finally, the *plateau* phase of action potential is shorter in immature cardiomyocytes because of the higher expression of Cav1.2 core component (CACNA1C) (98). Mature cardiomyocytes develop transverse-tubules (T-tubules), invaginations of the surface of cardiomyocyte's membrane that occur at the Z line, at the end of each sarcomere (99). Within mammalian cardiac tissue, T-tubules seems more present in ventricular myocytes than in atrial (100). An essential element for T-tubule organization is α -actinin-2 (87).

An adult human heart daily consumes an high amount of ATP produced by oxidative phosphorylation (101,102). The metabolic evolution from immature to mature cardiomyocytes is fueled by the activation of metabolic transcriptional regulators such as Ppargc1 α/β , Ppara α , Nrf1/2, and Esrra $\alpha/\beta/\gamma$ (103), leading to increased expression of genes involved in fatty acid metabolism, oxidative phosphorylation, and mitochondrial biogenesis and decreased expression of glycolytic genes (104,105). Also, during cardiac development changes in metabolism favour the switch from an immature to a mature isoform. Hexokinase isoform (HK1) is more abundant in fetal cardiomyocytes, replaced by HK2 with less glycolytic activity in late stage (106,107).

1.3.2. *In vitro* protocols for cardiomyocyte differentiation

The generation and use of animal and cellular models has paved the way to better understand how the cardiac lineage is defined in a stage-specific manner (i.e., from early stages of embryonic mesoderm commitment to the formation of embryonic and postnatal CMs) (108–110). The use of mice model system has allowed the definition of genetic programs, morphogens and signaling molecules determinant in cardiomyogenesis. However, mice models not fully recapitulate specific physiological, pathological and metabolic features of human biology and can be also debate for ethical issues (111,112). In this regard, the possibility to generate cardiac-like cells from hPSCs has helped researchers worldwide to better understand cardiac development in humans. Eventually, this information has now opened the door to the generation of model systems based in hPSCs-differentiation to better understand and target cardiac disease. In this section of the Introduction, information regarding seminal works describing the differentiation of hPSCs towards cardiac-like cells will be revisited. From strategies in 2D to the use of complex 3D culture set ups, this section aims to provide a better understanding of cardiomyocyte lineage specification and differentiation.

I. Cardiac differentiation from hPSCs in 2D

The differentiation in a 2D layer into cardiomyocytes is possible thanks to the subjection of hPSCs to various biochemical and physical cues, such as growth factors, small molecules inhibitors, and mechanical stimuli, mimicking an *in vivo* embryonic heart development. hPSC can be differentiated into CMs mediated 3 stages process involving spatial-temporal modulation of signaling pathways, such as BMP, activin-A, Wnt, etc. (113–115). In the recent past years, several protocols have been developed for the differentiation of hPSC into cardiac lineage. One of the first method described was the co-culture of PSCs and END-2 stromal cells (116). But the reported yield of cardiomyocyte generated using this protocol was low, with most cells displaying immature phenotypic characteristics. Nevertheless, this approach provided the basis for the fine tuning of the following methods. The next high impact protocol consisted into induction of EBs from PSCs grown in suspension (117). EBs are generated by growing PSCs into small-rounded aggregates that simulate the early stages of embryonic development. An advantage of this method was the possibility to

differentiate PSCs under fully defined conditions, but the disadvantages were the inconsistent range of success among experiments (5%-70%) in obtaining beating cardiomyocytes with low and immature-phenotype cardiomyocyte yield (113). For this reason, the urgent need to find alternatives for the generation of mature cardiomyocytes. Over the past years, an high number of 2D monolayer differentiation techniques have been developed with more mature and higher yield of cardiomyocytes (118). In 2007 Laflamme was the pioneer, who cultured hESCs on Matrigel in MEF-conditioned medium and at confluence switched to chemically defined RPMI-B27 medium supplemented with human recombinant Activin-A and BMP4 (113). Later, was discovered that Wnt/ β -catenin signaling is activated at the beginning of differentiation and inhibited when the mesoderm has developed (119). The comprehension of the role of this signaling pathway into cardiac commitment brings to the enhancement of all the following differentiation protocols. In 2012, one of the first protocols allowed the generation of high yield of mature cardiomyocytes in monolayer by applying the previous discoveries. It was added GSK3-inhibitor (CHIR) until the mesoderm formation and removed insulin from B27 medium during the first 5 days of differentiation (115,120). Together, all these data allowed the development of a more stable protocol for the differentiation of PSCs and it has been possible to implement it with the use of genetic engineering techniques and biomaterials (56).

II. Cardiac organoids

Undeniably, EBs and 2D cardiac differentiation have been until the recent years, a good model for development, disease and toxicology (121–123). Besides these advances, these models do not fully replicate the intricate structure of a 3D organ in terms of cell organization, morphology, and interaction with the ECM, among others. Major features of organoids refer to: cell heterogeneity, self-organization and recapitulation of specific functions similar to the organ *in vivo* (124). As mentioned in previous sections, up to day, a large variety of hPSC-derived organoids have been developed (e.g. lung, liver, brain, kidney) (27,38,39,125), but studies on the cardiovascular field are still improving (41,126–129). Among the different challenges that need to be overcome in the field of cardiac organoids, those refer to the need to develop different cell populations with complex functions, as well as the impossibility to mirror complex cellular environments organizing the heart tissue (i.e., mechanical and electrophysiological-relevant conditions, among many others).

Bioengineering approaches, such as the development of natural or synthetic scaffolds allowed the generation of artificially engineered heart tissues, referred also as cardiac organoids (130–133). Unluckily, these models fail to recapitulate cardiac-specific self-organization required for the acquisition of *in vivo*-like architecture and are restricted in their capacity to model human cardiogenesis and heart disease. In recent years, several protocols have been developed for the generation of more complex heart organoids. McDevitt's laboratory generated a multilineage organoid co-expressing mesoderm and endoderm cell lineages achieving the formation of complex organoids containing structures similar to the developing heart and gut forcing co-emergence of these unique populations during the time course of differentiation (129). Instead, Mendjan's laboratory established a cardiac protocol in 2D based on the temporal control of specific signaling pathway resulting into the specification of beating CM progenitors at above 90% efficiency (110). More recently, the same group took advantage of this protocol to stimulate the formation of complex 3D cardiac structures with cavity by supplementing the cell culture media with extracellular proteins involved in mesoderm development (128). At the same time, Aguirre's laboratory developed a procedure for the generation of heart organoids comparable to age-matched human fetal cardiac tissues by modulating Wnt signaling at early stages during the differentiation protocol. The authors showed that, by using this approach, cardiac organoids were capable of generating chamber-like structures while transcriptomically and structurally resembling the embryonic heart. Moreover, the authors exploited those culture systems to develop an *in vitro* model of pregestational diabetes (41). Another high-impact study was performed only a few months ago in Moretti's laboratory. They generated and characterized self-organizing hPSC-derived epicardioids displaying functional patterning from epicardium and myocardium by combining lineage tracing, single-cell transcriptomics and chromatin accessibility profiling (134). The above-mentioned protocols represent the first proof of concept towards the generation of complex cardiac organoids capturing complex features of the adult heart with the promise to further set up conditions to study heart development and disease.

1.4. Model systems to study kidney development and disease

Kidney disease is a health problem affecting millions of people worldwide. In the recent years, the prevalence of acute kidney injury (AKI) and chronic kidney disease (CKD) affect 10-15% of the population in several countries (135). Moreover, kidney injuries are often consequential complications derived from common health problems, such as diabetes, obesity, and cardiovascular disease (136). CKD can lead to end-stage renal disease (ESRD), where the only possible treatment is a kidney transplantation. Unluckily, there is a shortage of donor organs and the success of transplantation without rejection is very low. Therefore, there is an urgent need to develop novel regenerative medicine strategies for the treatment of kidney disease.

In this section of the Introduction different models leading to the generation of kidney-like cells and kidney organoids are going to be presented starting with a brief introduction on lessons learnt from animal model systems and how those findings are being translated into the field of biology through the use of hPSCs to understand and monitor these processes in the Petri dish.

1.4.1. *In vivo* kidney specification and differentiation

Kidney function consists, and is not limited to, filtering products and excess fluids from the blood that are then excreted through urine. Moreover, it helps regulate blood pressure, balance electrolyte levels, and produce hormones fundamental for bone health and red blood cell production. The understanding of kidney morphogenesis has been possible thanks to the isolation and growth *ex vivo* of this organ from different species. Using non-mammalian vertebrate species with simpler kidney, it has been possible to study the inductive signals and cellular processes that give rise to the mammalian kidney development.

I. Mammalian kidney development

In vertebrates, organ function is dictated from embryogenesis, except for kidney (137). Indeed, in mammals, the adult kidney is the outcome of 3-formation stages involving the maturation of 3 “different kidneys”: the pronephros, mesonephros and metanephros. Only the third one is the functional and definitive kidney (138,139). The pronephros development starts after gastrulation from the intermediate mesoderm

(IM), an embryonic germ layer derived from the primitive streak (PS), around E8 in mice (3rd week in human gestation). IM is an “intermediate” structure between the paraxial mesoderm (PM) and the lateral plate mesoderm (LPM) (140). As development carries on, the transient mesonephric kidney starts to form around E10.5 in mice (week 4 in human gestation), and the IM separates into anterior and posterior IM, which will specify into ureteric bud (UB) and metanephric mesenchyme (MM), respectively. The permanent kidney (metanephros) is made of collecting ducts (CDs) and nephrons, formed by the reciprocal interaction between UB and MM (141,142). Specifically, the UB gives rise to all the branches characterizing the collecting duct system (143), whereas MM differentiates to the stroma and cap mesenchyme (CM), the embryonic tissue from where all the epithelial cells along the nephron will arise. Finally, the CM will contain nephron progenitor cells (NPCs), cells undergoing to a mesenchymal-to-epithelial transition (MET) to generate the units of the kidney, nephrons, composed of more than 20 different specialized cells (144–146). In terms of time, the mature kidney begins to form at the 5th week of gestation in humans and become functional at the end of the 12th week (147) (Fig. 5).

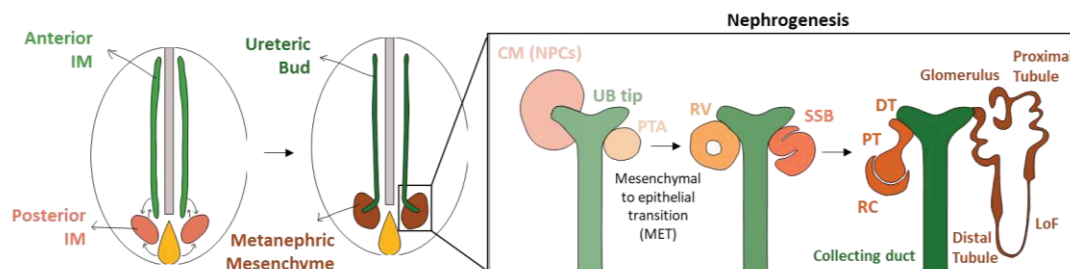


Fig. 5. Mammalian embryonic kidney development. After gastrulation, the intermediate mesoderm (IM), an embryonic germ layer situated between the lateral plate mesoderm (LPM) and the paraxial mesoderm (PM), is formed through the migration of cells from the primitive streak. The metanephros are derived from the interaction of progenitor cells from the anterior and posterior IM, which give rise to the ureteric bud (UB) and the metanephric mesenchyme (MM), respectively. Then, MM condenses to form caps around each UB tip forming the cap mesenchyme (CM) that is composed of active nephron progenitor cells (NPCs). Further condensation of the CM gives rise to the pre-tubular aggregate (PTA). PTA undergoes the process of mesenchymal-to-epithelial transition (MET) generating the renal vesicle (RV) [Figure from (148)].

II. Signaling pathways during kidney development

As mentioned above, the first primitive kidney generates from the IM, an embryonic germ layer derived from the PS. At transcriptional level, the IM is formed between the paraxial somatic mesoderm (PM) and the lateral plate mesoderm (LPM) thanks to the interaction between retinoic acid (RA), fibroblast growth factor (FGF) and Wnt signaling gradients. Brachyury (T) is the first mesodermal marker that identifies the PS fate (149). Subsequently, the posterior region of the PS gives rise to the IM which is characterized by the expression of the paired box protein 2/8 (Pax 2/8) (150,151), LIM homeobox 1 (Lhx1) (152), Odd-Skipped Related Transcription Factor 1 (Osr1) (153) and GATA3 (154,155). The IM receives both mediolateral patterning and anteroposterior patterning signals to specify the distinct kidney structures. While the anterior region of the IM will generate the UB, the posterior region of the IM will give rise to the MM.

As development continues, UB induction is driven by the expression of specific isoforms of homeobox paralogs (Hox) (Hoxb7 and Hoxd8) (141,156), the soluble growth factor glial-cell-line-derived neurotrophic factor (GDNF), secreted by the MM cells, and its receptor tyrosine kinase RET (157). GDNF/RET signaling is responsible for the proliferation and migration of the UB cells into the MM initiating the process of UB branching. Simultaneously Wnt9b secreted by the UB induces the expression of the nephrogenic markers Pax8, Lhx1, Wnt4 and Fgf8 in the MM which undergoes mesenchymal to epithelial transition (MET) to generate the nephron epithelia (nephrogenesis).

III. Nephron development (nephrogenesis)

E12.5 in mouse represents the stage of renal vesicle (RV) formation. From this moment, RVs evolve from the development of PTA which are in turn derived from the CM surrounding the tips of UB. In this process, Wnt pathway together with FGF8 and BMP7 signalling, play a crucial role (144,158). The NPCs generated at this stage through MET, are marked by the expression of the transcription factors SIX2 and CITED1 (159). Importantly, RV represent the first stage of nephron patterning and segmentation. Morphologically, nephron formation starts from RVs which latter transition into comma-shaped bodies (CSB) passing through further morphological changes until the acquisition of S-shaped bodies (SSB). At later stages of

differentiation, each SSB will develop into the epithelial portion of the nephron (from the glomerulus to the distal tubule). During all these transitions, the UB will give origin to the collecting duct system. It is well-known that both Notch and Wnt are determinant for RV establishment and transition into the SSB stage for the ulterior formation of podocytes, proximal tubules, and loop of Henle (160).

Other important cells during kidney development, are stromal progenitor cells. These cells are fundamental for NPCs regulation and UB development. Stromal cells produce RA, which contributes to UB branching and allow the upregulation of RET (161). Moreover, they give rise to the cells comprising the renal interstitium (glomerular mesangial cells, pericytes, among others), which is fundamental for ECM production and endocrine function (162) (Fig. 6).

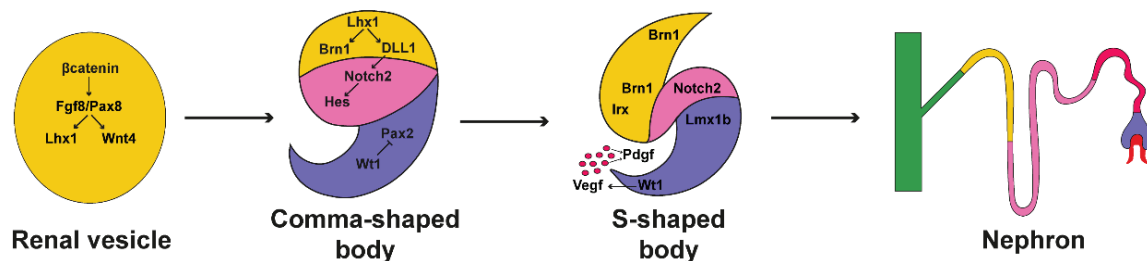


Fig.6. Key events of nephrogenesis. Wnt9 β , Pax8, Fgf8 and Wnt4 drive the formation of renal vesicles (RV). Wnt4 initiates the mesenchymal-to-epithelial transition (MET) in the MM giving rise to the formation of RVs. RVs polarize along the proximal/distal (or P/D) axis. Wnt- β catenin signaling activates Lhx1 that controls the expression of Brn1 and DLL1 in the distal precursors of the nephron, while the proximal nephron development depends on Notch signaling and is characterized by elevated Wt1 expression. Morphologically, the RV becomes the comma-shape body (CSB) that later transitions into the S-shape body (SSB). The distal portion of the SSB fuses with the collecting duct resulting into the formation of the patterned nephron [Figure adapted from (163)].

1.4.2. *In vitro* protocols for kidney differentiation from hPSCs

Kidney is a complex organ composed of many different cell types. The possibility of generating kidney-like cells and kidney organoids from hPSCs offers the opportunity to study kidney development and disease in the human context, and at the same time, opens the door to the potential development of new therapeutic options for the treatment of kidney disease. Nowadays, several *in vitro* protocols have been described to guide the differentiation of hPSCs into renal cell types and kidney organoids. These methodologies mainly rely on the intrinsic self-organization ability of hPSCs and the use of renal inductive signals known to be crucial during kidney development. In the

following paragraphs we will present the current knowledge on 2D and 3D differentiation procedures for generating renal cell types and kidney organoids from hPSCs.

I. Kidney differentiation from hPSCs in 2D

Early kidney differentiation protocols from hPSCs aimed to generate end-stage differentiated renal-like cell types. Indeed, previous studies reported the differentiation of hPSCs towards podocyte-like cells and proximal tubular-like cells by the exposure of hPSC monolayers to developmental factors such as activin A (AA), retinoic acid (RA), BMP2 or BMP7 (164,165). Those studies provided the first proof of concept that hPSCs can be induced towards the renal fate, one of the latest cell types that were generated from this unique cell source, even after haploid-like cells or gamete-like cells. Successively, Mae et al. redirected the focus of their studies on the early events of kidney development by generating OSR1- GFP reporter hiPSCs to monitor IM cells formation (166). Their renal differentiation approach consisted of 2 induction steps: first, using AA and CHIR treatment to induce mesendoderm fate (T^+ , GSC^+ and $MIXL1^+$ cells, meaning cells expressing these genes) and a second exposure to CHIR combined with BMP7 to promote the formation of cells positive for OSR1, PAX2, LHX1 and WT1. This approach satisfactorily induced the formation of IM-like cells, but unluckily could not reproduce efficiently ulterior cells from the IM (that is MM nor UB descendants). The dependance on CHIR as a first Wnt inducer during the first stages of renal differentiation was confirmed also in a successive study for the generation of mesendoderm layer (167). Moreover, the introduction of a new element, such as members of the fibroblast growth factor family (FGFs), led to the induction of IM-like cells, which was also used in ulterior studies on the derivation of renal tubular-like structures (168). In 2013, another approach used a chemically defined media supplemented first with BMP4 and FGF2 for mesoderm commitment, and then RA, AA and BMP2 for the generation of UB progenitor-like cells ($HOXB7^+$, RET^+ and $GFRA1^+$ cells). In this first study they generated chimeric kidney organoids for the first time through the aggregation of hPSCs-derived UB progenitor cells and mouse kidney embryonic stem cells exploiting the trans-well culture system (169).

Taken together, these findings laid the foundation for the establishment in the following years of renal differentiation methodologies to produce hPSC-derived kidney organoids in 3D culture.

II. Kidney organoids

Progress in understanding the mechanisms and signals that govern renal development has stimulated the development of different protocols for the generation of kidney organoids from hPSCs. Current hPSC-derived kidney organoid models offer a means to investigate human kidney development and disease.

To induce renal commitment from hPSCs, the field has relied on the use of different combinations of small molecules and growth factors that mimic the renal inductive signals occurring during kidney organogenesis. The first milestones in the field of kidney organoids occurred in 2015, when the laboratories of Nishinakamura (149), Little (170) and Bonventre (171) independently reported the generation of 3D kidney organoids from hPSCs. In the methodology established by the Nishinakamura laboratory, hPSCs were differentiated in 3D through the formation of EBs that were induced towards the posterior IM and subsequently to NPC by treatment with BMP, FGF, Activin and Wnt (149). hPSC-derived MM-like cells were subsequently co-cultured as cell aggregates with mouse embryonic spinal cords from E11.5 embryos at the air-liquid interface using a trans-well system to induce the MET, resulting in the formation of tubule-like structures containing distal and proximal nephron cell types. The Bonventre laboratory described the efficient generation of NPCs in only 9 days of 2D differentiation, that were subsequently aggregated into spheroids using a 96-multiwell 3D culture system. The induction of MET in the hPSC-derived NPC spheroids was achieved by treatment with CHIR promoting the formation of RV structures. Using this strategy kidney organoids with segmented nephron-like structures expressing markers of early podocytes, proximal tubules, loops of Henle and distal tubules were derived at day 35 of differentiation (171). Interestingly, in the methodology described by the Little laboratory, the authors could tailor the generation of UB and MM progenitors by adjusting the duration of CHIR treatment prior to FGF9 addition. They found that an intermediate duration of 4 days of exposure of hPSC monolayers to CHIR treatment simultaneously produced both anterior and posterior IM-committed cells. In the presence of FGF9 signaling, these cells were then forced to form aggregates that were cultured under organotypic culture conditions using a trans-well

system. After Wnt activation using CHIR, cell aggregates differentiated into kidney organoids containing segmented nephron-like structures (172), as similarly described by Nishinakamura and Bonventre's laboratories. Interestingly, kidney organoids derived following this methodology exhibited transcriptional similarities to the first-trimester human fetal kidney, as assessed by bulk RNA sequencing (172). Following a different 3D culture approach, Freedman et al. cultured hPSCs between 2 layers of Matrigel, referred to as "Matrigel sandwich culture system", to induce the formation of epiblast-like spheroids. Treatment of these spheroids with high dose of CHIR, to induce mesoderm specification and subsequent differentiation in the presence of FGF9 signalling to induce NPCs, allowed the formation of segmented nephron-like structures with podocyte-like cells, proximal tubules, and endothelial-like cells. However, neuron-like cell clusters were also detected surrounding the nephron structures (173).

In 2019, the host laboratory established a different procedure that showed the generation of kidney organoids transcriptomically matching second trimester human fetal kidney. By boosting cell-cell and cell-ECM interactions at early stages during NPC induction from hPSCs, kidney organoids containing segmented nephron-like structures were generated under 3D organotypic culture conditions in 16 days. Moreover, the generated kidney organoids were implanted onto the chick chorioallantoic membrane (CAM) for 5 days resulting in organoid engraftment and presence of host vascularization. Importantly, implanted organoids showed improved self-organization of the endogenous hPSC-derived endothelial-like cells, that were found in close contact to podocyte-like cells, and enhanced differentiation (39).

An alternative approach using transfection of synthetic mRNA (syn-mRNA) is from Hiratsuka et al. They showed that a consecutive transfection of 2 sets of synthetic mRNAs encoding transcription factors can induce differentiation of hPSCs into kidney cells with proximal and distant tubule cells and podocytes, but with low expression of segment-specific markers compared to other kidney organoids (174).

Thanks to the development of these methodologies, hPSC-derived kidney organoids have become a valuable research tool for studying kidney developmental processes and disease modelling. Nevertheless, hPSC-derived kidney organoid models still present several limitations including their insufficient maturation and the absence of relevant morphological characteristics, such as a proper ureteric tree connected to the nephrons, together with the formation of glomerular vascularization which is needed

for blood filtration. In this regard, recent efforts have started to combine hPSC-organoid technology with bioengineering approaches by making use of biomimetic biomaterials, micro/nanotechnology, 3D bioprinting, microfluidic devices and bioreactors to enhance the cellular and morphological complexity as well as maturation of different organoid models.

1.5. Genetically engineered hPSC-derived organoids

Currently, organoid model systems represent one of the most used *in vitro* platforms to study human development and disease. When derived from hPSCs, organoids exhibit important *in vitro* characteristics (i.e., possibility to culture them as single cells without losing their capacity to self-renew and differentiate, among many others) that enable genetic engineering, including the ability to add, replace, or remove parts of their genome. In this section of the Introduction several modalities of genome editing will be discussed, also highlighting their use in the context of hPSCs and hPSCs-derived organoids.

1.5.1. Genome editing in hPSCs

In mammalian cells, two machineries control the repair of the double stranded break (DSB): non-homologous end joining (NHEJ) and homology-directed repair (HDR). Biologically, NHEJ is preferred over HDR because is faster and active throughout cell cycle (175). But, at same time, NHEJ is error-prone because it repairs by adding or deleting bases without a homology template causing frameshift mutations giving rise to non-functional protein (in frame mutation or deletions-Indels). On the contrary, HDR is error-free because uses a homologous sequence derived from the sister chromatid as template for precise repair of cleaved DNA. Easy to guess that NHEJ is the mechanism used for the generation of knock-out, whereas HDR for knock-in and reporter lines (176).

The first breach in the wall for the birth of genome editing was in 1980, when Capecchi and Smithies discovered the natural mechanism of homologous recombination (HR) in eukaryotes (177,178). HR is the biological machinery working in the cells when DSB occurs and regions of homologous DNA exchange. This mechanism allowed the first

genetic modification of mESCs (179), but when it was translated to humans, an ample hurdle was found. The inefficiency derives from lower frequency of HR events in human cells. Later, pioneering experiments using biological restriction endonucleases from *Saccharomyces cerevisiae* (Sce-I) helped to overcome this hurdle, by highlighting that controlled DBSs induced by specific-cutting endonucleases can increase the efficiency of HR events in human cells (180). This breakthrough opened the door to the development of the new powerful approaches of genome editing based on engineered site-specific DNA nucleases that enable precise and programmable modification in hPSC genome (181). The most used genome-editing tools are zinc-finger nucleases (ZNFs), transcription activator-like effector nucleases (TALENs), and clustered, regularly interspaced, short palindromic repeats- CRISPR-associated 9 (CRISPR/Cas9). All of them are effective for genome editing in hPSCs, but it has been demonstrated that CRISPR/Cas9 works more efficiently. The 3 techniques are composed of a nuclease engineered to recognize, bind, and cut a specific sequence in the genome. ZNFs and TALENs are made up only of proteins, whereas CRISPR/Cas9 has protein and RNA components.

I. Zinc finger nucleases (ZFNs)

The first site-specific DNA nuclease engineered were the ZFNs. It is an artificial chimeric protein composed of a DNA-binding domain of a class of eukaryotic transcription factors, zinc finger protein (ZFPs), and Fok1, a cleavage domain derived from a monomer of a bacterial endonuclease (182,183). Each zinc finger DNA binding motif can recognize 3 bases, hence, in this model a chain of ZFPs are used to recognize longer and specific *loci* within the genome (184). At the same time, Fok1 to be catalytically active and cleave the site of interest needs to dimerize (185). Therefore, for gene targeting, a pair of ZNFs are used together to bind both DNA strands and cut the site of interest (186). However, this technology is quite challenging because of time-consuming and not easy manipulation.

II. Transcription activator-like effector nucleases (TALENs)

15 years after ZNFs were described, TALENs technology was developed. The principle is the same, both are chimeric proteins made of a modular DNA-binding domain fused to a Fok1 nuclease. The difference in TALENs consists in the transcription activator-like effector (TALE) component, discovered the first time in

Xanthomonas bacteria (187). TALEs are characterized by conserved domains of approximately 34 amino acids. This array of conserved domains only differs in the amino acids at position 12 and 13, called repeated variable diresidues (RVDs). RVDs define the DNA-binding specificity and they are responsible of DNA contact with a one-to-one correspondence (188), compared to ZNFs that recognize DNA triplets. For gene targeting, a tandem array of TALE repeats is engineered with RVDs of interest to target the site of interest (189). Although TALENs can be considered an upgrade over ZNFs for the less toxicity and time-consuming, there are still limitations also for this technique, such as cellular delivery for the large size and complex synthesis (190,191).

III. CRISPR/Cas9 system

In 2020 a women team composed by Doudna and Charpentier was awarded of the Nobel Prize in Chemistry for their innovative discovery in one of the greatest achievements in genetic technology: CRISPR/Cas9 technology (192). The term CRISPR, which stands for clustered regularly interspaced palindromic repeats, was used for the first time in 1987 as a series of short direct repeats interspaced with short sequences in the genome of *Escherichia Coli* by the Spanish scientist Mojica (193), Cas (CRISPR-associated) is a gene encoding for proteins with nuclease domains. Later, CRISPR/Cas were detected in numerous bacteria as defense mechanism against foreign DNA (194). Among all of them, 3 types (I-III) of CRISPR systems have been identified. CRISPR/Cas9 for genome editing is an adaptation of the immune system found in the bacteria *Streptococcus pyogenes* using the type II CRISPR system, one of the best characterized (192). Specifically, the type II consists of the nuclease Cas9, one CRISPR RNA (crRNA) containing nuclease guide sequences, and another noncoding RNA, known as trans-activating crRNA (tracrRNA). When a foreign DNA (i.e., virus) attacks for the second time the bacterium, crRNA:tracrRNA recognizes the invasion and recruits Cas9 endonuclease to cut and silence the viral gene (195). The translation of these elements to mammalian cells made the genome editing of easy access. Another important advantage of this system is that RNA-guided Cas9 system recognizes its target sequence only when this is immediately followed by a protospacer-adjacent motif (PAM) sequence at the 5' end (196). In this way, a single guide RNA (sgRNA) has been engineered by fusing the crRNA and tracrRNA to create a chimeric sgRNA and Cas9 can be directed toward any target of interest

around the PAM sequence by modifying the 20-nt guide sequence within the sgRNA. In particular, Cas9 performs the cleavage 3bp upstream of the PAM motif (NGG) (192,197). When DSBs occurs in the target gene, cells can activate or NHEJ resulting in random indels (knock-out mutations) or HDR, if a homologous DNA template is provided (knock-in mutations) (195). The easy engineering of nucleases, higher efficiency and specificity, and low cost have made the CRISPR/Cas9 system the most widely used genome editing tool in recent years, over ZFNs and TALENs. (198).

In 2014, an improvement of this genetic engineering technique was achieved when Gonzalez et al. were able to perform multiplexing targeting in hPSCs (199). The advantages of this genome editing platform, named iCRISPR, were not only the integration of a stable Cas9 cassette and the possibility to transfect small sgRNAs, but above all, the introduction of a tetracycline-inducible Cas9 expression module, which allowed for the generation of single and multiple inducible gene knock-out and knock-in. Specifically, in their experimental set up, the Cas9 expression cassette is integrated into the human safe-harbor *AAVS1 locus*, via 2 donor plasmids by TALEN-mediated targeting: the Neo-M2rtTA donor plasmid (coding for a constitutive reverse tetracycline transactivator) and the puro-Cas9 donor plasmid (containing the tetracycline-inducible Cas9 expression cassette). Thanks to the introduction of the TET-ON system, the addition of doxycycline (DOX), an analogue of tetracycline, allows M2rtTA to bind to the tetracycline responsive element (TRE) promoter and to activate Cas9 expression (200,201). Although iCRISPR brought big advances in the field of genome editing in hPSCs, there were still some technical limitations: the occupancy of both alleles of the *AAVS1 locus* which limited further genetic engineering, and antibiotic resistance to Neomycin and Puromycin.

1.5.2. Generation of reporter cells lines in combination with hPSC-derived organoids

The knowledge achieved in the derivation of protocols directed to recapitulate human organogenesis from hPSCs has been boosted by the exploitation of genetic engineering techniques, such as generation of reporter cell lines as powerful tool monitoring and improving differentiation cardiac and kidney processes *in vitro*. Here

we are going to review early works exploiting on this approach to better understand differentiation into the cardiac and renal lineage.

I. Generation of reporter cells lines to understand cardiac development

One of the major limitations in the field of hPSCs differentiation is the generation of off-target populations. A way to overcome this issue has been the introduction of fluorescent reporter proteins by labelling specific genes of interest, such as cardiac genes. Above all, this approach helps to decipher key transition stages essential for cardiogenesis while helping to improve their generation *in vitro*. A reporter cell line can be generated in a transient or stable manner. In the first approach, the transgene remains episomal, whereas for the generation of stable reporter cell lines, the integration of the fluorescent reporter takes place through its integration in the host genome. In both cases, the fluorescent protein is inserted downstream of a promoter or gene of interest (GOI) (202). The development of site-specific endonucleases, above all CRISPR/Cas9 technology, allowed to overcome the low efficiency of transient approaches as well as the limitations due to random integration when using viral vectors for the introduction of fluorescent markers. When revisiting the different approaches undertaken for the derivation of cardiac reporter cell lines, we need to go back to the last 20 years, finding early studies deriving reporter PSCs lines (starting with ESCs) for cardiac developmental studies, as well as other approaches allowing for cardiac cell isolation and functional studies. Several methods have been employed to create cardiac reporter cell lines, but the most widely utilized technique thus far has been the direct transduction of a plasmid containing a reporter gene under the control of the desired promoter through electroporation. In 1996, Metzger et al. engineered a mESC in a way to express the *LacZ* gene under the transcriptional control of α -actin promoter (203). Later, it was established a similar approach with a GFP as reporter gene (204). During cardiac commitment, one of the earliest cardiovascular progenitor markers identified in gastrulation is *T*, which as mentioned above, is highly expressed in early mesoderm and down-regulated when cells undergo ulterior differentiation towards more differentiated progenies. To monitor the developmental events involved in the induction and specification of mesoderm, *T locus* was targeted with a GFP reporter first in mESCs (205,206). As already mentioned, inhibition and activation of Wnt pathway drive the transition from early mesoderm towards cardiac mesoderm.

Reporter cell lines for the transcription factor mesoderm posterior basic helix-loop-helix transcription factor 1 (MESP1) and NK2 homeobox 5 (NKX2.5) helped the identification of these intermediate cells (207). In this regard, in 2008 Hsiao et al. described a method to create mESCs NKX2.5_2AemGFP reporter cell line by Bacterial Artificial Chromosome (BACs) recombineering (208). More specific cardiac lineages are defined by the FHF and SHF, the first one forming the left ventricle and the SHF generating right ventricle, outflow tract and inflow tract, respectively. An early study exploited the insulin gene enhancer protein (ISL1) as a marker for SHF through the generation of a hESC reporter cell line (209). Thus, other cardiac specific markers have been chosen for more effective targeting. Among others, MYH6 has been largely explored as a good candidate for the generation of cardiac reporter cell lines. As previously stated (see section 1.3.1), it is expressed at early and late stages during cardiac differentiation also exhibiting an essential role for cardiac muscle contraction. One of the first approach for the generation of this reporter cell line was in mESCs in which a GFP expression was driven by a short form from a α -MHC promoter sequence (210). In the latest years, attempts have been made to increase the number of reporter cell lines generated in hPSCs. In 2009, Kita-Matsuo et al. generated lentiviral vectors combining T or α -MHC promoter sequences with mCherry or GFP fluorescent proteins and antibiotic resistance under the promoter of Rex1 (211). Then, one of the first human α -MHC-GFP reporter cell line was generated by Ritner et al. in 2011. Following a similar strategy, the host laboratory made use of TALEN mediated genome editing to introduce the mCherry fluorescent reporter in the MYH6 of hESCs to efficiently follow cardiomyocyte-like cells differentiation from hPSCs and repopulate decellularized human heart ventricles for heart engineering (56). More recently, reporter cell lines generated using latest CRISPR/Cas9 genetic engineering techniques have been exploited. Overall, reporter cell lines have opened a new door for the monitoring of cardiac differentiation essential during the modelling of cardiac disease and the generation of high throughput cardiac protocols.

1.6. Organoids to model human disease

One of the major goals for researchers has always been to find the cellular and molecular pathophysiology mechanisms underlying diseases. Early studies focused

on analyzing *post-mortem* pathological samples before advancing to the utilization of animal models. Animal models, ranging from the fruit fly *Drosophila* and the worm *C. Elegans* to vertebrate models, such as the mouse and the zebrafish, have allowed us to elucidate the role of specific genes during embryonic development. However, animal models cannot fully recapitulate the (patho)-physiology and function of the human tissues. In the last decades, the development of 3D tissue-like structures derived from hPSCs recapitulating the architecture and function of human organs, represents the most powerful model for the study of human-related diseases. Overall, the use of organoids to model human disease holds great promise for advancing our understanding of the underlying systemic conditions that contribute to diseases. In the following sections, we will discuss the use of cardiac and kidney organoids for disease modelling applications.

1.6.1. Cardiac organoids as human disease model

Nowadays, the leading cause of death worldwide and the most common type of congenital defects in humans are cardiovascular diseases and congenital heart defects, respectively. Hence, the generation of human heart organoid represent a useful model for the study of the molecular pathology underlying cardiac diseases. Cardiac microtissues engineered from hiPSCs derived from patients affected by dilated cardiomyopathy (212) and hypertrophic cardiomyopathy (213) initiated the comprehension of specific diseases related to heart. Cardiac organoids exposed to local tissue damage using cryoinjury by dry ice was able to model acute myocardial infarction (AMI) showing an accumulation of extracellular matrix proteins (128,214). Moreover, the combination of organoids model with CRISPR/Cas9 genome editing made possible the generation of disease-carrying mutations in healthy cells or to rescue patients' cells in organoids. In 2021, the generation of knock-out (KO) HAND1 and NKX2.5 hESC line for modelling human cardiac malformations demonstrated that HAND1-KO and NKX2.5-KO resulted in a reduced size of cavity and cardiac organoids (128). Another NKX2.5-KO hESC line was generated in the same year by Drakhalis et al., showing a similarity to NKX2.5 KO mice (215) with a reduction in tissue compaction and adhesion between cardiomyocytes (126). The abovementioned achievements emphasize the potential of organoid technologies and the means by

which developments in human cardiac disease organoid models may facilitate the way for breakthroughs in cardiac drug discovery (216).

1.6.2. Kidney organoids as human disease model

Chronic kidney disease (CKD) affects 10% of the worldwide population and is the cause of millions of deaths every year. Therefore, there is the need to develop models that can help the comprehension of pathogenesis underlying CKD. The use of hPSCs and, more recently, the development of protocols for the generation of 3D kidney organoids, have opened a new door for the modeling of human kidney disease *in vitro* and the improvement of new therapeutic strategies.

Generation of genetically modified kidney organoids allowed the study of tubular and glomerular genetic kidney diseases (170,217,218). Polycystic kidney disease (PKD) is one of the most prominent tubular diseases due to autosomal dominant mutations in the polycystin-1 and -2 genes (219–221). The knock-out of these genes in hiPSC lines using the CRISPR/Cas9 system and the further generation of kidney organoids can faithfully mimic the cystic phenotype found *in vivo* in diseased patients (173,217). In another work, kidney organoids generated from hiPSC derived from patients with mutations in the tuberous sclerosis complex-2 gene underwent to a mutation correction through CRIPR/Cas9 and exhibited reduced cyst formation compared to the affected organoids (222).

On the glomerular side, kidney organoids derived from patient-derived mutant NPHS1 hiPSCs have been used as model for abnormal podocytes formation (170). Such as PODXL-KO hiPSC line used for the generation of kidney organoids showed faulty podocyte architecture (173). In conclusion, these studies highlight the importance of utilizing genetically engineered hPSC-derived kidney organoids to gain a deeper understanding of the molecular mechanisms that govern human renal diseases.

1.6.3. Modeling systemic conditions with cardiac and kidney organoids

A systemic disease is characterized by a disorder affecting multiple organs and tissues, or even the entire body. While diseases confined to a single organ may be

easier to study, it is important to acknowledge the existence of systemic disorders and the need to model them *in vitro* to gain a comprehensive understanding of their development and to facilitate the progress of effective treatments. One of the most common multi-systemic diseases is diabetes. Diabetes mellitus is characterized by high glucose levels (hyperglycaemia) due to not sufficient insulin production (type 1), body insulin-resistance (type 2) or hyperglycaemia during pregnancy (gestational diabetes). Diabetes can bring to secondary complications (microvascular and macrovascular) affecting several organs. Here we would like to focus our attention on heart and kidney diabetic-related damages, better-known as diabetic cardiomyopathy (DC) and diabetic nephropathy (DN), respectively. As it is tough to recapitulate systemic disorders *in vitro*, organoids can be a powerful accessible model for DN and DC. DC is present in diabetic patients with an abnormal cardiac structure in absence of other cardiac risk factors (223). To model DC using hPSCs, it is necessary to supply the cells to the same environment found in the diabetic state. One of the pioneering experiments was performed in 2D cell cultured derived from hiPSCs-CM and showed the induction of diabetic changes by culturing the cells in media containing fatty acids without glucose and media with intermediate glucose levels, endothelin 1 and cortisol. These cells expressed hallmarks of diabetes, such as glucose restriction-induced cellular distortion, lipid accumulation and peroxidation, decreased frequency of calcium transients, cellular hypertrophy, and loss of sarcomere integrity (224). Successively, Graneli et al. showed mitochondrial dysfunction, typical in diabetes, with an increase in basal respiration and decrease in maximal respiration by using first linoleic and oleic acids without glucose to induce insulin resistance in hiPCS-CM and then, high levels of glucose and palmitate supplemented with uric acid and endothelin-1 (225). Another approach for insulin-resistance induction was established by Fialho et al. by culturing hiPSC-CM for a week in maturation media supplemented in a first stage with 5mM glucose, oleic acid, then glucose-free, palmitic acid and insulin, and the last step with 12 mM glucose (226). The only existing 3D cardiac cell model for diabetes until now is the one developed by Lewis-Israeli et al. They modelled the effects of pre-gestational diabetes by exposing cardiac organoids to high glucose levels and different concentration of insulin. Diabetic organoids showed arrhythmia, lower oxygen consumption, reduction in mitochondrial number and size and cardiac hypertrophy (41).

On the kidney side, DN is the leading cause of end-stage renal disease (ESRD) characterized by persistent albuminuria and a progressive decline in the glomerular filtration rate. To date, there are no effective treatments, and the need of a good preclinical model is high. The host laboratory mimicked a diabetic *milieu* in kidney organoids from hPSCs (39) by supplementing the media with continuous low glucose (5 mM, control) and high glucose in an oscillatory fashion (5-25 mM, diabetic). Diabetic organoids showed transcriptional changes, ECM alterations and early diabetic hallmark (227). Moreover, this model showed also that these oscillating glucose concentrations bring to a metabolic programming in the kidney that increases SARS-CoV-2 infection susceptibility compared with control. This is one of the few kidneys organoid model existing to model DN. Therefore, the urgent need to generate more complex cardiac and kidney organoids models to gain better insight on the mechanisms governing human systemic diseases, such as diabetes.

OBJECTIVES

2. OBJECTIVES

The general aim of this thesis is the definition of new cell culture conditions that promote cardiac and renal lineage differentiation, providing valuable insights into organ development and disease modeling. To pursue this goal, we harnessed the power of organoid technology and pioneered the development of innovative biomaterials. Through these efforts, we worked on the establishment of advanced hPSC-derived biomodels, serving as invaluable tools for conducting promising research in the field of regenerative medicine applications.

To achieve the general aim, the specific objectives of the thesis are:

1. To generate hPSC cardiac reporter lines mirroring the endogenous expression of MYH6, MYL2, and SIRPA genes taking advantage of a recently developed hPSC platform named iC2.
2. To characterize the generated hPSC cardiac reporter lines following a well-established cardiac differentiation procedure developed previously in the host laboratory.
3. To define new cell culture conditions to promote 3D differentiation from hPSC cardiac reporter lines to establish cardiac-like organoids.
4. To develop an isogenic platform for the study of early hallmarks of diabetic disease exploiting cardiac-like organoids.
5. To produce organ-derived hydrogels based on decellularized extracellular matrices from heart and kidney tissues.
6. To set up new conditions for the generation of hPSC-derived kidney and heart organoid systems based on the use of biomimetic materials defined in 5).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Cell culture and maintenance of hPSCs

The approval of the Ethics Committee of the Center of Regenerative Medicine in Barcelona and the Comisión de Seguimiento y Control de la Donación de Células y Tejidos Humanos del Instituto de Salud Carlos III for the use of the hPSCs to be used in this thesis refers to several project listed here as project numbers: 0336E/7564/2016; 0336E/5311/2015; 0336E/15986/2016; 0336E/79489/2015; 00336E/20031/2014; 0336/1123/2021 0336/214/2022;9067/487286/2022. In this thesis we have used two hPSC lines expressing an inducible form of Cas9 (iC2 lines) (148,229). These lines (female and male backgrounds) were generated through the targeting of one allele of the *AAVS1 locus* via TALEN genetic engineering with an inducible Cas9 cassette, where Cas9 gene transcription was under the control of the TET/ON system. These lines have been generated in the context of different projects in the host laboratory and at the present time work is conducted towards their registry at Banco Nacional de Líneas celulares from the Insitute of Health Carlos III and the European registry hPSCreg.

Depending on the experiment, all cell lines used in this thesis were routinely cultured in two hPSC specific media supplemented with 1% Penicillin/Streptomycin (P/S, 10.000 U/mL, Thermo Fisher Scientific, 15140122). In particular, all differentiation experiments were performed with cells cultured in mTeSR medium (StemCell, 85850 Technologies) on Matrigel (Cultek, 45354277), while all the genome editing experiments were performed in Essential 8 medium (E8, Life Technologies, QA1517001) on vitronectin coating (Life Technologies, A14700). The hPSC cultures were passed when they reached the 80% confluence, generally every 5-7 days, using 0.5 mM EDTA (Thermo Fisher Scientific, 15575-038) by detaching them into small clusters. Briefly, after removing the medium, the cells were rinsed once with 1X PBS (Thermo Fisher Scientific, 10010056) before adding the 0.5 mM EDTA solution. The cells were then incubated for 3 min at 37 °C. Afterwards, EDTA was aspirated, and the cells were recovered with the medium by gently flushing. The cell clumps were collected in a 15 mL tube and the volume of medium was adjusted to perform the adequate dilution (generally 1:6) for passage.

3.1.1. Cryopreservation and thawing

For storage of hPSCs, cells were detached in small clusters, as described in the previous section, collected in a 15 mL tube, centrifuged for 5 min at 300 g and resuspended in pre-chilled freezing medium, containing 90% medium and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650) as cryoprotectant. Generally, we froze 1-2 million of cells per mL of freezing medium in one Nunc™ cryogenic tube (Thermo Fisher Scientific, 375418). Cryogenic tube with cells were then transferred to a Mr. Frosty™ freezer container (Thermo Fisher Scientific, 5100-0001), overnight at -80°C, to allow the freezing of cells at the ideal speed of -1 °C per minute. For long term storage, the frozen vials were then transferred to a liquid nitrogen container.

For thawing of cryopreserved cells, stored vials were recovered from the liquid nitrogen container and kept on dry ice until they were quickly placed in a water bath at 37 °C for 30 sec. To ensure good viability after thawing, the manipulation of the frozen vials needed to be fast. Hence, 1 mL of thawed cells were quickly transferred from the cryotube to a 15 mL tube containing 9 mL of pre-warmed medium. The cells were pelleted by centrifuging for 5 min at 300 g, resuspended in fresh medium and plated in 3 wells of pre-coated 6-multiwell plate.

3.2. Generation of hPSC reporter lines

Using both iC2 hPSCs lines, we generated different hPSC reporter lines mirroring the endogenous expression of cardiac genes, important for lineage specification and function, including: MYH6, MYL2 and SIRPA.

The generation of each reporter cell line required the nucleofection of 3 elements:

- a sgRNA (sections 3.2.1 and 3.2.2).
- a donor vector (section 3.2.3).
- a puromycin bait vector (section 3.2.4).

3.2.1. sgRNA design and synthesis

Genomic sequences of targeted genes were downloaded from Ensembl genome browser (<http://www.ensembl.org>) and saved in a sequence analysis software

(MacVector), followed by the annotation of all exons. 100 nucleotides (nt) of sequence around the stop codon were used as query in a sgRNA design tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM). The 3-4 highest-ranking sgRNAs per gene, with the highest on-target and lowest off-target predicted activity, were selected for further functional testing in iC2 lines.

Selected sgRNAs were ordered and purchased as 2 nmol Alt-R® CRISPR-Cas9 crRNAs (Integrated DNA Technologies, IDT) and annealed with Alt-R® CRISPR-Cas9 tracrRNA (IDT) following the manufacturer's guidelines. In Table 1 the selected sgRNAs used for each hPSC reporter cell line:

Table 1: Custom Alt-R CRISPR-Cas9 crRNAs	
crRNA name	crRNA sequence
Cas9.MYH6repCr1	TGGCAAGAGTGAGGTTCCCG
Cas9.MYH6repCr2	GATGAGGAGTGACACTGCCT
Cas9.MYH6repCr3	ATGAGGAGTGACACTGCCTC
Cas9.SIRPArepCr1	ACGGTCCCA TTCACTTCCTC
Cas9.SIRPArepCr2	CCATTCACTTCCTCGGGAAC
Cas9.SIRPArepCr3	ATGGGTGCTAGAGCAAACCA
Cas9.SIRPArepCr4	CACGGTCCCA TTCACTTCCT
Cas9.MYL2repCr1	ACTCTGCAAAGACGAGCCCA
Cas9.MYL2repCr2	CACTCTGCAAAGACGAGCCC
Cas9.MYL2repCr3	CTAGTCCTTCTCTTCTCCGT

3.2.2. sgRNA efficiency assessment screen: TIDE analysis

To functionally test all selected sgRNAs, iC2 hPSCs line was previously treated with 2 µg/mL Dox (Thermo Fisher, BP26535) for 24 h and with 10 µM ROCK inhibitor (Sigma, Y-0503) for 2h. According to the manufacturer's guidelines, for each sgRNA, we prepared the transfection complex containing:

- 1 µl of 20 µM crRNA/tracrRNA duplex
- 3 µl of RNAiMAX reagent (Thermo Fisher, 13778075)
- 96 µl of OptiMEM medium (Gibco, 31985047)

Briefly, after removing the medium, the cells were rinsed twice with 1X PBS before adding TrypLE Express reagent (Thermo Fisher, 12604021) to detach them in single cells. After the incubation at 37 °C for 4 min, TrypLE was aspirated, and the cells were dissociated gently, but thoroughly with E8 medium without P/S, containing 2 µg/mL Dox and 10 µM ROCK inhibitor. The single cell suspension was passed through a 40 µm cell strainer (Cultek, 45352340) and the cells were counted with Countess II FL counter (Thermo Fisher Scientific, AMQAF1000). The cells were resuspended at 200.000 cells/mL of E8 medium without P/S supplemented with 2 µg/mL Dox and 10 µM ROCK inhibitor and seeded at 200.000 cells/well of VTN pre-coated 12 multiwell plate. 24h after transfection, the medium was changed and replaced with fresh E8, supplemented with 2 µg/mL of Dox for a further 24 h. When the cells reached 80% of confluence, we collected them for genomic DNA extraction and analysis. Briefly, after removing the medium, the cells were rinsed once with 1X PBS, mechanically detached from the plate by using a cell scraper and 1mL of PBS. Then, the cells were pelleted, and genomic DNA isolated by using DNeasy Blood and Tissue kit (Qiagen, 69504) according to the manufacturer's guidelines (Section 3.6.1). To quantify the percentage of all insertions or deletions (Indel) in the target sequence for each sgRNA, we generated 2 PCR amplicons (~700 bp stretch of DNA inclusive of the target *locus*), both from the same primer set, to amplify the genomic target sequence of the control sample (DNA from wild-type cell without sgRNA) and the genomic target sequence of test sample (DNA from a pool of cells expressing Cas9 and transfected with sgRNA). We used Herculanase II Fusion DNA Polymerases (Agilent, 600679) to generate all PCR fragments. PCR products were then purified with NucleoSpin Gel and PCR Cleanup (Macherey-Nagel, 740609,50) and sequenced. Two primers for Sanger sequencing were designed, ~200 bp upstream and downstream the projected cut site (Table 2).

To quantify the editing efficacy for each transfected sgRNAs, we used the TIDE web-tool (<https://tide.nki.nl/>). The inputs to TIDE are the Sanger sequencing data from the control and the test sample. The outputs of TIDE are the comprehensive profiles of all insertions and deletions in the edited sample, mediated by NHEJ. The region upstream of the cut site is used to align the sequencing data of the test sample with that of the control sample.

Table 2: Oligos for TIDE		
Oligo name	Sequence Forward	Sequence Reverse
MYH6_TIDE_PCR	GTAGGCAGTTCTGAGGGTCC	GGCAGAACACCTCACTCCTC
MYL2_TIDE_PCR	CCGGAGGACCCTCTGAGT	GGAACATGGCCTCTGGATGG
SIRPA_TIDE_PCR	CCGAGAGGACACAGAAGCAT	CGTCAGCCTCCAGGTATGTG
MYH6_TIDE_SEQ	GCTTACATAACCTGAGAATC	CCTTGTGGATATGGGATG
MYL2_TIDE_SEQ	CAGGTTGACCAGATGTTC	GAAGATGGAGGTGGATAA
SIRPA_TIDE_SEQ	GACTTGGTATTCAGTTTGAG	CAGGTATGTGGCAATGTG

3.2.3. Generation of reporter donor plasmids

The generation of all reporter cell lines making use of our recently developed iC2 platform (148,229), here we took advantage of the possibility to push HDR in the genome after Cas9-mediated DBS. To this aim, we generated specific reporter donor vectors to fill the gap in the genome with an exogenous homologous DNA template. We used the HiFi DNA Assembly (NEB, M5520A), for the cloning of multiple DNA fragments amplified by PCR, to generate each specific donor vector. In particular, each donor vector contained a 5' homology arm (5HA), the HA epitope followed by the T2A fragment, the GFP or the mOrange fluorescent gene, followed by a 3' homology arm (3HA). 5HA and 3HA are a ~600 bp stretch of gene sequence flanking the stop codon (Table 3).

To prevent multiple genome editing events, we introduced silent point mutations in the homology arms of each donor vector to eliminate the PAM sequence. If the modifications of the DNA sequence were not possible without any change at protein level, we modified the sequence of the protospacer in the donor vector to eliminate the complementarity between the sgRNA and the genome.

Table 3: Oligos for cloning MYH6 mOrange and MYH6 GFP	
Oligo name	oligo sequence
<i>pDonor_MYH6_5HA_F</i>	GCATTTATCAGGGTTGAAATTCGGATGAGGCAGAGGAGCG
<i>T2A_HA_MYH6_5HA_R</i>	CGCCGCTGCCAGCGTAATCTGGAAACATCGTATGGGTACTCCTCATCGTGCAATTTTTG
<i>Universal_HA_T2A_Reporter_F</i>	AGATTACGCTGGCAGCGGCGAGGGCAGG
<i>MYH6_3HA_Reporter_R</i>	CGAGTCAGTGTTACTTGACAGCTCGTCCATGCC
<i>Reporter_MYH6_3HA_F</i>	GTACAAGTAACACTGACTCGGGAACTCTCACT
<i>pDonor_MYH6_3HA_R</i>	TTTACGGTTCTGGCGAAATTCCTCAAAAAGAAATGGGTGTCCTAGGA
Oligos for cloning MYL2 mOrange	
Oligo name	oligo sequence
<i>pDonor_MYH6_5HA_F</i>	GCATTTATCAGGGTTGAAATTCCTTGAATTTGTGATCCCATGTG
<i>T2A_HA_MYH6_5HA_R</i>	CGCCGCTGCCAGCGTAATCTGGAAACATCGTATGGGTAGTCTTCTCTCTCCGTGAGTGATGATGTGCAACC
<i>Universal_HA_T2A_Reporter_F</i>	AGATTACGCTGGCAGCGGCGAGGGCAGG
<i>MYH6_3HA_Reporter_R</i>	GAGCCCTCTTACTTGTAACAGCTCGTCCATGCC
<i>Reporter_MYH6_3HA_F</i>	GTACAAGTAAGAGGGGGCTCGCTGCTGCGCTCTGGGCTCGTCTTTG
<i>pDonor_MYH6_3HA_R</i>	TTTACGGTTCTGGCGAAATTCCTATGTTCTCAATGTGTCAAGTCC
Oligos for cloning SIRPA mOrange	
Oligo name	oligo sequence
<i>pDonor_SIRPA_5HA_F</i>	GCATTTATCAGGGTTGAAATTCGGTGAAGACTGCAGGTGG
<i>T2A_HA_SIRPA_5HA_R</i>	CGCCGCTGCCAGCGTAATCTGGAAACATCGTATGGGTACTTCTCTCGGACCTGGAACG
<i>Universal_HA_T2A_Reporter_F</i>	AGATTACGCTGGCAGCGGCGAGGGCAGG
<i>SIRPA_3HA_Reporter_R</i>	ACGTTCCCATTTACTTGTAACAGCTCGTCCATGCC
<i>Reporter_SIRPA_3HA_F</i>	GTACAAGTAAATGGGAACGTGTTTGCTC
<i>pDonor_SIRPA_3HA_R</i>	TTTACGGTTCTGGCGAAATTCGCTTTTCCATGGAAAGGGGACG

3.2.4. Generation of pRR-Puro bait vectors

For all sgRNAs used, we generated a unique pRR-Puro vector by cloning the corresponding protospacer-PAM sequences between a prematurely terminated 5' sequence of the puromycin resistance gene and a full-length puromycin resistance coding sequence without the start codon. The pRR-Puro vector was constructed through the *SacI*-*AatII* cloning of the protospacer-PAM fragment, into pRR-Puro plasmid (Addgene, 65853). The specific double strand protospacer-PAM fragment was assembled through annealing of 2 single strand oligos (Table 4).

Table 4: Oligos for protospacer-PAM fragment	
Oligo name	oligo sequence
MYH6_SIRPA_MYL2_F	CTGCAGATGAGGAGTGACACTGCGCTCGGGCCGAGGAAATGTGAAATGGGACCGTGCCTGGGCTCGTCTTTGCAAGTAGT
MYH6_SIRPA_MYL2_R	ACTCTGCAAAAGACGAGCCGAGGGGCGCGTCCCATTCACCTTCTCGGCCGAGGCAAGTGTCATCTCTCATCTGCAAGCT

3.2.5. Nucleofection of plasmids and sgRNA

Before nucleofection, iC2 hPSCs lines were cultivated in E8 medium complemented with 2 µg/mL Dox for 24 h and with 10 µM ROCK inhibitor for 2 h. Then, the cells were dissociated using TrypLE for 4 min at 37 °C, collected in E8 medium, complemented with 2 µg/mL Dox and with 10 µM ROCK inhibitor. To remove possible clumps and ensure a single cell solution, collected cells were filtered through a 40 µm cell strainer. The cells were rinsed with 1X PBS, centrifugated at 300 g for 5 min and counted. For nucleofection, we used the Amaxa Nucleofector II device and Human Stem Cell Nucleofector Kit 1 (Lonza, VPH-5012). After setting up cell culture conditions $1,5 \times 10^6$ or 3×10^6 undifferentiated iC2 hPSCs were used for subsequent nucleofection depending on the specific cell background. Total cells were resuspended in 100 µL of Nucleofection mix, containing 82 µL of Nucleofection solution and 18 µL of supplement, which were later transferred into a 1.5 mL tube containing 3 µg of donor vector, 2 µg of pRR-Puro vector and 18 µL of 20µM of Alt-R crRNA/tracrRNA duplex. The whole mixture was placed in a nucleofection cuvette and electroporated using the Nucleofector™ program B-016. This program was selected based on optimization experiments previously performed in the laboratory. Immediately after, the nucleofected mixture was carefully transferred with Amaxa plastic pipettes into a 15 mL tube containing 10 mL of E8 medium supplemented with 2 ug/mL Dox and with 10 µM ROCK inhibitor. After pelleting (3 min at 300 g), the cells were resuspended in 10 mL of E8 medium supplemented with 2 ug/mL Dox and with 10 µM ROCK inhibitor and seeded in a VTN-coated p10 culture dish. The day after, the medium was changed with fresh E8 and cells were cultivated for 48 h before treatment with 0,33 µg/mL of Puromycin Dihydrochloride (Thermo Fisher Scientific, A1113803) for 4 days. After antibiotic selection, 96 transiently resistant colonies were randomly picked, amplified, replicated, stocked and DNA was extracted for PCR genotyping, following the PCR protocol with Herculase II (See section 3.6.4) (Table 5).

Table 5: Oligos for genotyping reporters			
Oligos for genotyping MYH6 mOrange and GFP <i>loci</i>			
Oligo name	Oligo sequence	PCR	Expected size (bp)
Genotyping_MYH6_F	GGACTCTGAGTGCTGGACAT	w t PCR	1354 (mOrange) 1353(GFP)
Genotyping_MYH6_R	GCCCCAGAGTCTCAAGATGC		
Genotyping_MYH6_F	GGACTCTGAGTGCTGGACAT	5H PCR	821
Universal_Genotyping_R	CTCCTCGCCCTTGCTCAC		
Universal_Genotyping_F	GGCATGGACGAGCTGTACAA	3H PCR	665
Genotyping_MYH6_R	GCCCCAGAGTCTCAAGATGC		
Oligos for genotyping MYL2 mOrange <i>loci</i>			
Oligo name	Oligo sequence	PCR	Expected size (bp)
Genotyping_MYL2_F	GCCACGTGTCTCCATTTAGC	w t PCR	1343
Genotyping_MYL2_R	GTCAGTACCACTCCCTCAGTTT		
Genotyping_MYL2_F	GCCACGTGTCTCCATTTAGC	5H PCR	781
Universal_Genotyping_R	CTCCTCGCCCTTGCTCAC		
Universal_Genotyping_F	GGCATGGACGAGCTGTACAA	3H PCR	694
Genotyping_MYL2_R	GTCAGTACCACTCCCTCAGTTT		
Oligos for genotyping SIRPA mOrange <i>loci</i>			
Oligo name	Oligo sequence	PCR	Expected size (bp)
Genotyping_SIRPA_F	GCACAAAGAAGGAACATGGACC	w t PCR	1389
Genotyping_SIRPA_R	CCCAAGGAAGCAGAGGGTTT		
Genotyping_SIRPA_F	GCACAAAGAAGGAACATGGACC	5H PCR	826
Universal_Genotyping_R	CTCCTCGCCCTTGCTCAC		
Universal_Genotyping_F	GGCATGGACGAGCTGTACAA	3H PCR	695
Genotyping_SIRPA_R	CCCAAGGAAGCAGAGGGTTT		

3.3. Differentiation of hPSCs towards cardiac-like cells in 2D and 3D

3.3.1. Differentiation of hPSCs towards cardiac-like cells in 2D

Differentiation of hPSCs reporter cell lines towards cardiac-like cells in 2D was performed by using well-established protocol from the host laboratory (56).

On day -2 (*D*-2), hPSCs at 80% confluency with big and compact colonies were used to start the differentiation protocol. Cells were pre-treated 2 h before starting the differentiation protocol with 10 μ M ROCK inhibitor. After 2 h of ROCK inhibitor, cells were detached using Accumax (Stem Cell Technologies, 07921) and incubated for 5 min at 37 °C to disaggregate the cell clumps into a single-cell suspension. Single- cells were collected in mTeSR medium and centrifuged at 1200 rpm for 5 min to remove Accumax. The pellet of single- cells were resuspended in 5 mL of mTeSR medium. From this suspension, 200 μ L were mixed with 200 μ L of Trypan blue solution 0,4%

(Thermo Fisher Scientific, 15250061), from which 10 μ L were placed on a Countess™ cell counting chamber slide for automated cell count in Countess™ II FL Automated Cell Counter. Based on viability and cell number, the collected single- cells were resuspended in the appropriate volume of mTeSR medium to have 1×10^6 viable cells/mL. Single-cell suspension of hPSCs were seeded onto matrigel pre-coated cell culture dishes at a density of 375.000 cells per cm^2 in mTeSR medium. Cells were maintained in mTeSR for 48 h. On *D0*, differentiation was initiated by treatment of undifferentiated hPSCs with 10 or 12 μ M CHIR99021 (Sigma, SML1046) depending in the specific cell background. From that moment mTeSR medium was replaced by RPMI 1640 (Thermo Fisher, 11875-093) supplemented with B27 minus insulin (Thermo Fisher, A1895601), 2mM GlutaMAX (Thermo Fisher, 35050061), 0.1mM 2-mercaptoethanol (Thermo Fisher, 31350010), nonessential amino acids (Cultek, BE13-114E) and penicillin-streptomycin (Thermo Fisher, 15140122) [RPMI/B27-insulin medium] for 24 h (*D0* to *D1*). On *D1* ROCK inhibitor was removed. On *D3*, cells were treated with 5 mM Wnt inhibitor IWP4 (Stemgent, 24804-0036) in RPMI/B27-insulin medium and cultured without medium change for 48 h. From *D5* on, RPMI/B27-insulin medium was replaced with RPMI/B27+insulin medium, replacing B27 minus insulin with B27 (Thermo Fisher, 12587-010) and this medium was maintained until *D30* with medium change every 2 days. On *D12*, beating monolayers were obtained.

3.3.2. Differentiation of hPSCs towards cardiac-like organoids

Differentiation of hPSC reporter cell line 1 towards cardiac-like organoids was performed the same as the 2D protocol previously explained until *D3*. On *D3*, for cardiac-like organoids generation, cell monolayers were detached in single- cells using Accumax. The plate was incubated for 3 min at 37 °C before removing Accumax. Then, single- cells were collected in 5 mL of RPMI/B27-insulin medium. For cell counting, 200 μ L of the single-cell suspension were mixed with 200 μ L of Trypan blue solution, from which 10 μ L were placed on a Countess™ cell counting chamber slide for automated cell count in Countess™ II FL Automated Cell Counter. Based on viability and cell number, cells were resuspended in the appropriate volume of medium to have 1×10^5 viable cells for 100 μ L of RPMI/B27-insulin medium supplemented with 5 mM Wnt inhibitor IWP4 and 5 μ M ROCK inhibitor. To generate the organoids, 100 μ L of the adjusted cell suspension were pipetted in each well of a conical-bottom 96-

well plate (Thermo Fisher Scientific, 249935), which was afterwards centrifuged for 3 min at 300 *g*. The 96-well plate was then maintained in an incubator at 37 °C with 5% CO₂ for 48 h without medium change to allow cells to self-aggregate into a free-floating spheroid. From *D5* on, RPMI/B27-insulin medium was replaced with RPMI/B27+insulin medium, replacing B27 -insulin with B27 and this medium was maintained until *D90* with medium change every 2 days. On *D10*, beating cardioids were obtained.

3.3.3. Glucose challenge in cardiac-like organoids

Normoglycemic conditions were emulated exposing cardiac-like organoids to DMEM glucose-free (Thermofisher, 11966025) supplemented with 5 mM D-Glucose (Merck, G7021) changing media every day from *D21* to *D28* or from *D62* to *D70*. Diabetic-like condition involved the exposure of cardiac-like organoids to DMEM glucose-free supplemented with 5mM D-Glucose (5 mM glucose medium) on even days and with 25 mM D-Glucose (25 mM glucose medium) on odd days from *D21* to *D28* or from *D62* to *D70*. 5 mM glucose medium was additionally supplemented with 20 mM D-Mannitol (M9647, Merck) to obtain the same molarity as in the 25 mM glucose medium.

3.4. Differentiation of hPSCs towards kidney organoids

The protocol for hPSC differentiation into kidney organoids was performed as indicated in the previous publication of the lab (39).

On *D-2*, hPSCs that have grown up to 80% confluency and have big and compact colonies were used to start the differentiation protocol. Cells were detached in colonies using EDTA 0.5mM in E8 media. 100 µL of the cell suspension were mixed with 300 µL of Accumax and incubated for 5 min at 37 °C to disaggregate the cell clumps into a single-cell suspension for cell counting. From this suspension, 50 µL were mixed with 50 µL of Trypan blue solution 0,4%, from which 10 µL were placed on a Countess™ cell counting chamber slide for automated cell count in Countess™ II FL Automated Cell Counter. Based on viability and cell number, the collected cell clusters were resuspended in the appropriate volume of E8 media to have 4x10⁵ viable cells/mL. Afterwards, to have a starting plating density of 2x10⁵ cells/well of a VTN-

coated 24-well plate (Thermo Fisher Scientific, 142475), 500 μ L of the resultant cell suspension were plated after aspirating the VTN coating. Cells were cultured at 37 °C with 5% CO₂ for 48 h before starting the differentiation. Once cell colony density and distribution were adequate on *D0*, the E8 media was replaced by differentiation basal media supplemented with 8 μ M CHIR. The differentiation basal media was composed of advanced RPMI 1640 basal media (Thermo Fisher Scientific, 12633-012) supplemented with 1% GlutaMAX and 1% Penicillin/Streptomycin (10.000 U/mL). Cells were cultured for 3 consecutive days with 8 μ M CHIR treatment to achieve the posterior primitive streak (PPS)-related fate. Media change was performed daily to refresh the 8 μ M CHIR treatment. On *D3*, once cultured cells showed an appearance of dense clusters, the media was replaced by differentiation basal media supplemented with 200 ng/mL FGF9 (Peprotech, 100–23B), 1 μ g/mL heparin (Sigma-Aldrich, H3149-10KU) and 10 ng/mL activin A (Sigma-Aldrich, 338-AC-050). On *D4*, the cell monolayer was incubated for 1h with 5 μ M CHIR treatment in advanced RPMI 1640 basal media, supplemented also with 200 ng/mL FGF9 and 1 μ g/mL heparin. Following the CHIR pulse, cells were rinsed with 1X PBS and 200 μ L of TrypLE were added to each well to enzymatically detach the cell monolayer. The plate was incubated for 1 min at RT before removing the TrypLE. Then, cells were collected in a 15 mL tube using 500 μ L/well of advanced RPMI 1640 basal media supplemented with 200 ng/mL FGF9 and 1 μ g/mL heparin, and the total volume was gently pipetted up and down 2-4 times to ensure the obtention of a single-cell suspension. For cell counting, 50 μ L of the single-cell suspension were mixed with 50 μ L of Trypan blue solution, from which 10 μ L were placed on a Countess™ cell counting chamber slide for automated cell count in Countess™ II FL Automated Cell Counter. Based on viability and cell number, cells were resuspended at 10×10^5 to in 100 μ L of differentiation basal medium supplemented with 200 ng/mL FGF9 and 1 μ g/mL heparin. To generate the organoids, 100 μ L of the adjusted cell suspension were pipetted in each well of a conical-bottom 96-well plate, which was afterwards centrifuged for 3 min at 300 *g*. The 96-well plate was then maintained in an incubator at 37 °C with 5% CO₂ for 48 h without medium change to allow cells to self-aggregate into a free-floating spheroid and induce the differentiation towards nephron progenitor cells (NPCs). From *D7* to *D11*, media changes were made every 48 h with differentiation basal medium with growth factors (200 ng/mL FGF9 and 1 μ g/mL heparin). From *D12* onwards, the media change was made every 48 h with

differentiation basal medium without growth factors. After 24 h of growth factors removal, nephrogenesis led to the appearance of multiple renal vesicles (RVs) -the precursor structures of the nephrons- within the spheroid (RV-stage organoid). The differentiation of this RV-stage organoid over the following days led to the formation of fully developed kidney organoids by *D20*, which contained nephron-like structures with podocyte-like and tubular-like cells.

3.5. Nucleic acid manipulation and processing

3.5.1. Genomic DNA extraction

To extract and purify genomic DNA (gDNA) from cells and organoids, the DNeasy Blood & Tissue Kit (Qiagen, 69504) was used following manufacturers' protocol for "Cultured cells". This kit was used for both hPSCs and organoids.

Pellets of up to 5×10^6 hPSC cells or approximately 12 pooled organoids were collected in a 1.5 mL tube and washed once with 1X PBS to remove the culture media. Dry pellets were then stored at -20 °C or immediately used for DNA extraction. All the reagents mentioned were supplied by the Qiagen kit. Samples were resuspended in 200 μ L of 1X PBS with 20 μ L proteinase K. Then 200 μ L of AL buffer was added for lysis incubation at 56°C for 10 min. Addition of 200 μ L of ethanol 100% cancelled the lysis reaction. After mixing thoroughly by vortexing, the entire volume was transferred into the DNeasy Mini spin column placed in a 2 mL collection tube to bind the DNA to the membrane. The columns were centrifuged at $\geq 6000 g$ for 1 min, after which the flow-through and the column were discarded. The columns were then placed in new collection tubes and same procedure was performed consecutively with 500 μ L of AW1 buffer and, afterwards, with 500 μ L of AW2 buffer as washing steps for the membranes. Lastly, 2 elution steps were performed for maximum DNA yield in Nuclease-Free Water (Thermo Fisher Scientific, AM9937). Eluted DNA was then stored at -20 °C or directly used for further applications.

3.5.2. RNA isolation

For the extraction of total RNA from cells and organoids, the method used was guanidinium thiocyanate-phenol-chloroform extraction, using the TRI Reagent

(Sigma-Aldrich, T9424). Pellets of cells or approximately 12 pooled organoids were collected in a 1.5mL tube, washed once with 1X PBS to remove the culture media, and resuspended in 1 mL of pre-chilled TRI Reagent. Samples in TRI Reagent could be stored at – 80 °C or continued to be processed. To ensure the extraction of high-quality RNA, samples were always kept on ice and RNase-free material was used to avoid RNA degradation.

Cell pellets were easily mechanically disaggregated by pipetting. On the other hand, organoids required mechanical disaggregation with a 29G syringe (BD, 320924). Next, 200 µL of chloroform (Sigma-Aldrich, 1.02445) were added and samples were mixed thoroughly by vortexing. After incubating 2 min at RT, samples were centrifuged at 16,000 *g* during 20 min at 4 °C for organic and aqueous phase separation. At this point, 3 phases were clearly observable: the lower organic phase corresponding to phenol-chloroform, the interphase, and the upper clear aqueous phase containing the RNA. Hence, the upper aqueous phase (approximately 500 µL) was recovered and placed in a new 1.5 mL tube. To precipitate and isolate high yields of RNA, the same volume of ice-cold isopropanol (Panreac, 131090.1612) was added, and samples were incubated O/N at – 80 °C. The following day, samples were allowed to thaw on ice and then centrifuged at 16,000 *g* during 60 min at 4 °C. The supernatant was discarded, and the RNA pellets were washed with 1 mL of 70% ethanol (Merck, 1.00983). Samples were mixed thoroughly by vortexing and centrifuged at 16,000 *g* during 10 min at 4 °C. After ethanol removal by carefully pipetting, RNA pellets were air-dried for 10 min to remove residual traces of ethanol. Then, RNA pellets were resuspended with Nuclease-Free Water and left on ice for a few hours to increase solubility. RNA quantification was preferably performed before storage at – 80 °C.

3.5.3. Nucleic acid quantification

The concentration and purity of the isolated DNA and RNA were determined with Nanodrop™ 2000 (Thermo Fisher Scientific). For measuring, 1 µL of the nucleic acid sample was placed onto measurement pedestal. Concentration was expressed in ng/µL and the purity was determined by the ratio of 260nm/280nm absorbances. A ratio of approximately 1.8 for DNA and 2.0 for RNA was considered of good quality

and indicated low presence of contaminants (proteins, phenol, etc.) that could interfere in downstream applications.

3.5.4. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) technique was performed using Herculase II Fusion Enzyme with dNTPs Combo (Agilent, 600679), following manufacturer's indications. Generally, the PCR reaction was set as follows:

Table 6: PCR reaction mix	
Component	Volume of component (μL)
5X Herculase II reaction buffer	2
100 mM dNTPS (25 mM each)	0.1
10 μM primer mix	0.5
Herculase II Fusion Polysmerase	0.1
Nuclease-Free H ₂ O	6.3
Template DNA (50 ng gDNA, 1 ng plasmid DNA)	1
Total volume	10

Depending on the number of samples, a master mix was prepared with all the components and then 9 μL of the mix were combined with 1 μL of the template DNA in a 0.2 mL PCR tube (Biologix, 60-0008). Samples were placed on a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, A24811) and the cycling conditions were generally set up as follows:

Table 7: Thermal cycling program for PCR reaction			
Step	Temperature	Time	Number of cycles
Initialization	95 °C	3 min	1x
Denaturation	95 °C	30 s	35x
Annealing	60-65 °C (depending on primer T _m)	30 s	
Elongation	72 °C	30 s/kb of target	
Final extension	72 °C	3 min	1x

Primers' sequences and products' sizes are specified in the sections where PCR technique was used (Section 3.3.5).

3.5.5. Agarose-gel electrophoresis

The products of the PCR reactions were subjected to electrophoresis in agarose gels, which allowed to separate nucleic acids by charge and size. Generally, 1% agarose gels were prepared by dissolving 1 g of AgaPure™ Agarose LE (Canvax Biotech, AG006) in 100 mL of 1X TAE buffer. The solution was heated until no lumps of agarose were observed. Once the solution was slightly cooled, 10 µL of SYBR Safe (Thermo Fisher Scientific, S33102) were added to allow staining and visualization of the PCR products. Once mixed, the agarose solution was poured in a cast to allow polymerization at RT and a comb was introduced to generate the wells of the gel. The solidified agarose gel was placed in an electrophoresis chamber filled with 1X TAE buffer. To determine the size of the nucleic acid fragments, either GeneRuler 100 bp Plus DNA ladder (Thermo Fisher Scientific, SM0322) or GeneRuler 1 kb Plus DNA ladder (Thermo Fisher Scientific, SM0311) were used as molecular weight markers. The samples were prepared with 6X DNA Gel Loading Dye (Thermo Fisher Scientific, R061). Electrophoresis run at 120 V for approximately 30 min, and the bands were visualized using the Gel Doc™ XR+ Molecular Imager (Bio-Rad).

Note: 50X TAE buffer was prepared dissolving 242 g of TRIZMA base (Sigma-Aldrich, T1503) in 700 mL of MiliQ H₂O. Then 57.1 mL of 100% glacial acid and 100 mL of 0.5 M EDTA (pH 8.0) were added, and final volume was adjusted to 1 L.

3.5.6. Reverse transcription PCR (RT-PCR)

The reverse transcription PCR (RT-PCR) is a variation of the PCR reaction that converts and amplifies total RNA into complementary DNA (cDNA), which can be later used for quantification of mRNA expression. In this work, RNA was retro-transcribed using iScript™ Reverse Transcription Supermix (Bio-Rad, 1708841BUN), according to manufacturer's instructions. Generally, ~ 1 µg of total RNA was retrotranscribed with the following reaction:

Table 8: RT-PCR reaction mix	
Component	Volume of component (μL)
iScript RT Supermix	4
RNA template (100 ng/μL)	10
Nuclease-Free H ₂ O	6
Total volume	20

Reactions prepared in 0.2 mL PCR tube (Biologix, 60-0008) were placed on a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, A24811) and the cycling conditions were set up as follows:

Table 9: Thermal cycling program for RT-PCR reaction		
Step	Temperature	Time
Priming	25 °C	5 min
Reverse transcription	46 °C	20 min
RT inactivation	95 °C	1 min

The resulting cDNA was ~ 50 ng/μL, and it was further diluted to 6.25 ng/μL for performing Real Time quantitative PCR (RT-qPCR).

3.5.7. Real Time quantitative PCR (RT-qPCR)

In this work, Real Time quantitative PCR (RT-qPCR) was used for quantification of mRNA expression for measuring levels of gene expression of defined targets. Hence, specific primers were designed for amplification of mRNA by selecting the forward and reverse primer sequences from different but consecutive exons. This approach avoids amplification of genomic DNA and therefore, misinterpretation of gene expression analysis. Primers were designed with annealing temperatures of ~ 60 °C and amplified products from 60 to 120 bp. A final 4 μM primer mix was prepared from combining 100 μM forward and reverse primers. For RT-qPCR, the PowerUp SYBR® Green Master Mix (Thermo Fisher Scientific, A25778) was used. The SYBR® Green dye detects PCR products by emitting fluorescence upon binding to all double-stranded DNA formed during PCR amplification. As the PCR reaction amplifies the products, the

increase in fluorescence intensity is proportional to the amount of PCR products generated. The quantitative output of RT-qPCR is the threshold cycle (Ct), which is defined as the PCR cycle at which fluorescence crosses an arbitrary threshold, normally set at the exponential phase of the PCR amplification. Relative gene expression is then determined with the $2^{-\Delta C_t}$ calculation, in which the expression of a gene of interest is normalized to the expression of a housekeeping control gene.

The RT-qPCR reaction for detection of expression of specific genes was set up as follows:

Table 10: RT-qPCR reaction mix	
Component	Volume of component (μL)
PowerUp SYBR® Green Master Mix	5
cDNA (6.25 ng/μL)	4
4 μM primer mix for each specific gene	1
Total volume	10

The reactions were set in technical duplicates in MicroAmp™ Optical 384-Well Reaction Plate (Thermo Scientific, 4309849). For running the RT-qPCR reaction, the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, A28140) was used. The design of the plate setup, the running protocol and the analysis mode were selected with the Design and Analysis Software of the Thermo Fisher Connect™ platform. The template used was “Quantification-SYBR-with_Melt” and the cycling conditions were the following:

Table 11: Thermal cycling program for RT-qPCR reaction			
Step	Temperature	Time	Number of cycles
Hold	95 °C	10 min	1x
PCR	95 °C	15 s	40x
	60 °C	1 min	
Melt Curve	95 °C	15 s	1x
	60 °C	1 min	
	95 °C	15 s	

The result of the analysis was expressed as “expression of the gene of interest relative to expression of housekeeping gene”. The housekeeping genes used were *GAPDH* and *RPLP0*. The graphs were generated using GraphPad Prism version 9.0.0 software.

The RT-qPCR primers used are described in the following table:

Table 12: RT-qPCR primer sequences

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>RPLP0</i>	GGTGTAATCCTGCATGGCTG	AGCAAGTGGGAAGGTGTAATCC
<i>GAPDH</i>	AGCAATGCCTCTGCACCAACAAAC	CCGGAGGGGCCATCCACAGTCT
<i>Oct-04</i>	AGTGAGAGGCAACCTGGAGA	ACACTCGGAACACATCCTTC
<i>ACLY</i>	CATATCCAGAGGAAGCCTACATTG	ATGGTCCAGATCCTCCCTTT
<i>ACOX2</i>	CGGTGAAGTCAAAAGCATCA	AAAGTGACATGGCAAGAACA
<i>ACSS2</i>	TTGGGGCTTTGCACTCCATT	AGGCATCTGTAGTGATGAGAAGA
<i>ATP11A</i>	CCCAGAGTCGTGAGGAATCG	CGTCTTTCACTGGACGGT
<i>BRACHYURY</i>	GCAAAAGCTTTCCTTGATGC	ATGAGGATTTGCAAGGTGGAC
<i>CACNA1C</i>	GAAGCGGCAGCAATATGGGA	TTGGTGGCGTTGGAATCATCT
<i>CD31</i>	GAAAGCTGTCCCTGATGCCG	CCATCAAGGGAGCCCTCCG
<i>COL3A1</i>	AGGACTGACCAAGATGGGAA	AGGGGAGCTGGCTACTTCTC
<i>COL4A1</i>	CCTTTTGTCCTTCACTCCA	CTCCACGAGGACACAGC
<i>CPT1A</i>	GGCTGAGCCATGAAGCTCTTA	GGGTACACGCCAGTGATGAT
<i>CPT2</i>	CACCATGCACTACGAGGACAG	CCTGAACTGGCCATCATTCAA
<i>CRIPTO</i>	CGGAACTGTGAGCACGATGT	GGGCAGCCAGGTGTCAATG
<i>DNMT3b</i>	GCTCACAGGGCCCGATACTT	GCACTCCTGCAGCTCGAGTTTA
<i>ENDOGLIN</i>	CCTACGTGTCTGGCTCATC	GGTGTGTCTGGGAGCTTGAA
<i>FN1</i>	CTGGCCAGTCCTACAAACAG	CGGGAACTCTCTCTGTACGCC
<i>GATA3</i>	CGTCTGTGCGAACTGTCA	GTCCCCATTGGCATTCCTCC
<i>GATA4</i>	GGGACGGGTCACTATCTGTG	GGTGGTGGTCTGGCAGTT
<i>GDNF</i>	CCAAACCCAGAGAAATCCAGA	AGCCGCTGCAGTACCTAAAA
<i>HK2</i>	AGCCCTTTCTCCATCTCCTT	AAACCATGACCAAGTGACAGAA
<i>KCNA4</i>	TTCCTACACGGATCTGCTGC	AGCGTAGGCTGACACATTT
<i>KCNH2</i>	TTCGACCTGCTCATCTTCGG	CGATGCGTGAGTCCATGTGT
<i>KCNJ2</i>	CCTGGCTTTGCTCTGTCAAT	TGAAGCTGTTGACCTCGGAC
<i>KCNQ1</i>	AACTCATGGTGCGCATCA	CCGCGATCCTTGCTCTTTT
<i>KCNQ2</i>	GGAAACCGTTCTGTGTGATTGAC	TCCGCAGAATCTGCAGGAA
<i>LDHA</i>	GGAGATCCATCATCTCTCCC	GGCCTGTGCCATCAGTATCT
<i>MYH6</i>	GACTGTTGTGGCCCTGTACC	GGAAGGATGAGCCCTTTTTC
<i>MYL2</i>	CGCCAACTCCAACTGTGTTCT	CCATCCCTGTTCTGGTCCAT
<i>NFAT1C</i>	CCTGTCCCTCACTGCTCTACA	GCACTCTCAATCCGAAAGCTCA
<i>NPHS1</i>	GGCTCCCAGCAGAAACTCTT	CACAGACCAAGCAACTGCCTA
<i>OSR1</i>	CTGCCCAAACCTGTATGGTTT	CGGCACCTTTGGAGAAAGAAAG
<i>PAX2</i>	CCCAAAAGTGGTGGACAAGAT	GAAAGGCTGCTGAACTTTGG
<i>PAX8</i>	GGCTCCACCTCATCCATCAA	CTGCTGCTGCTCTGTGAGTC
<i>PODXL</i>	GATAAGTGCGGCATACGGCT	GCTCGTACACATCCTTGGCA
<i>PPARα</i>	ATGGTGGACACGGAAAGCC	CGATGGATTGCGAAATCTCTTGG
<i>PPARδ</i>	TGAACGCAGATGGACCTCTA	TCAACACAGTGGCTTCTGCTC
<i>PPARγ</i>	ACCAAAAGTGCAATCAAAAGTGA	ATGAGGGAGTTGGAAGGCTCT
<i>REX1</i>	CCTGCAGGCGGAAATAGAAC	GCACACATAGCCATCACATAAGG
<i>SCNA5</i>	TCTCTATGGCAATCCACCCCA	GAGGACATACAAAGCGTTGGT
<i>SERCA2</i>	ATGCACCTTGAGGACTCTGC	ATCATGATGACCCGGATGCC
<i>SIRPA</i>	CGAGGACGTTCACTCTCAAGTCA	GAACTCGGATGGTCTCAGACAAAG
<i>SIX2</i>	GGCCAAAGGAAAAGGAGAACA	GAGCTGCCTAACACCGACTT
<i>SLC3A1</i>	CACCAATGCAGTGGGCAAT	CTGGGCTGAGTCTTTTGGAC
<i>TNNT2</i>	CAAAAGCCAGGTGTTTCAATG	CCGGTGGATGTATCAAAAGTC
<i>TNNI3</i>	CCAACTACCGCGCTTATGC	CTCGCTCAGCTCTTGCTTT
<i>UCP2</i>	GAACGGGACACCTTTAGAGAAG	CAGCAACAAGACGAGATAGAGG
<i>VIMENTIN</i>	GTTGACAAATGCGTCTCTGG	TGTTCTGAACTGTAGCCTG
<i>WT1</i>	GCGGAGCCCAATACAGAAATA	GATGCCGACCGTACAAAGAGT

3.5.8. Sanger sequencing

To validate the success of genome editing, a PCR surrounding the target sequence of positive clones was performed and underwent Sanger sequencing for the Genomic facility of the Parc Científic Barcelona (PCB) (Table 13).

Table 13: Oligos for Genotyping		
Oligo name	Sequence Forward	Sequence Reverse
Seq_MYH6_Cr1_2_3	GCTTACATAACCTGAGAATC	CCTTGTGGATATGGGATG
Seq_MYL2_Cr1_2_3	CAGGTTGACCAGATGTTC	GAAGATGGAGGTGGATAA
Seq_SIRPA_Cr1_2_3_4	GACTTGGTATTTCAGTTTGAG	CAGGTATGTGGCAATGTG

3.6. Electron Microscopy

Specimens were fixed with 2.5% glutaraldehyde containing 1% tannic acid in 0.1 M phosphate buffer (PB, pH 7.4). Then samples were post-fixed for 1 h at 4°C with 1% OsO₄ in 0.1 M PB. Epoxy resin embedding was performed after graded ethanol series. Then toluidine blue staining was performed in semithin sections using a light microscope. Finally, ultrathin sections were prepared using an EM UC7 ultramicrotome (Leica Microsystems, Mannheim, Germany) and collected on copper grids. Then 4% uranyl acetate and lead citrate were used for staining. Samples were subsequently analyzed with a JEM 1230 electron microscope (JEOL, UK, Ltd).

3.7. Histological staining

Paraffin-embedded organoids sections (3–5 µm in thickness) were air-dried and further dried at 60°C overnight. Histochemical analyses were carried out for hematoxylin–eosin (HE) and Masson Trichrome (MT) using an Artisanlink Pro machine (DAKO AR165 kit). Immunohistochemical assays were performed using an Autostainer Plus (Dako—Agilent). MT images were acquired with a NanoZoomer-2.0 HT C9600 digital scanner (Hamamatsu) equipped with a 20X objective.

3.8. Immunocytochemistry (ICC)

In this thesis, immunocytochemistry (ICC) was performed to analyze the extent of differentiation of the cardiac-like and kidney organoids by detection of proteins expressed at the different stages of cardiac and nephron development.

3.8.1. Immunocytochemistry in 2D

Paraffin sections and monolayer differentiation were washed with 1X PBS and fixed for 30 min with 4% paraformaldehyde (PFA) (1:4 dilution of 16% PFA, ANAME, 15710-S) for kidney and 2% PFA for cardiac tissue/differentiations. Samples were washed for 5 min 3 times with 1X PBS to remove PFA excess. For blocking, samples were incubated 1 h at RT with 1X TBS containing 0.5% Triton X-100 (Sigma-Aldrich, T8787) and 3% donkey serum (Sigma-Aldrich, S30-KC). For detection of proteins of interest, samples were incubated O.N. at 4°C with primary antibody solution diluted in 1X TBS containing 0.1% Triton X-100 and 3% donkey serum. After primary antibody incubation, samples were washed with 1X TBS containing 0.1% Triton X-100 and 3% donkey serum 3 times for 10 min each at RT. The corresponding secondary antibodies solution was prepared also in 1X TBS containing 0.1% Triton X-100 and 3% donkey serum for 2h at RT. After secondary antibody incubation, samples were washed with 1X TBS three times for 10 min each at RT. To stain the nuclei, samples were then incubated with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Invitrogen, D1306) at a 1:2500 dilution in 1X TBS. Samples were mounted on a microscope glass slide (Thermo Fisher Scientific, J1800AMNZ) and Fluoromount-G (Southern Biotech, 0100-01). Samples were visualized using a Super-resolution Inverted Confocal Microscope Zeiss LSM 880-Airyscan Elyra PS.1 and Confocal Microscope Zeiss LSM 800.

3.8.2. Immunocytochemistry in 3D

For each time point, organoids were collected in a 1.5 mL tube, washed with 1X PBS and fixed O/N at 4°C with 1 mL of 4% PFA for kidney and 2% PFA for cardiac organoids. The next day, organoids were washed for 15 min 3 times with 1X PBS to remove PFA excess.

In each staining, at least 2 organoids were placed in a well of 24-well plate. For blocking and permeabilization, organoids were incubated O/N at 4°C with 500 µL of 1X TBS containing 1% Triton X-100 and 6% donkey serum. For detection of proteins of interest, organoids were incubated with 250 µL of primary antibodies solution for 48-72 h at 4°C. Primary antibodies were diluted in 1X TBS containing 0.5% Triton X-100 and 6% donkey serum. After primary antibody incubation, organoids were washed with 500 µL of 1X TBS containing 0.5% Triton X-100 and 6% donkey serum 3 times for 30-60 min each at RT in a rocking platform. The corresponding secondary antibodies solution was prepared also in 1X TBS containing 0.5% Triton X-100 and 6% donkey serum, and organoids were incubated O/N at 4°C in 250 µL of this solution. For nephron structure analysis in which the biotinylated Lotus Tetragonolobus Lectin (LTL) was used to stain proximal tubule-like structures, an additional blocking step with the Streptavidin/Biotin blocking kit (SP-2002, Vector Labs) was required to block the organoid endogenous biotin. Briefly, 4-5 drops of streptavidin solution were used to incubate the organoids for 60 min at RT. Same additional procedure was performed for Wheat Germ Agglutinin (WGA) to stain T-tubule presence. Afterwards, samples were washed once with 1X TBS for 15 min at RT in the case of organoids. The next step was to use 4-5 drops of biotin solution for a new 60 min incubation, and it was finished with a one wash with 1X TBS for 15 min at RT. After using the Streptavidin/Biotin blocking kit, the solutions to dilute primary and secondary antibodies contained 1% BSA instead of donkey serum. After secondary antibody incubation, samples were washed with 500 µL of 1X TBS 3 times for 30-60 min each at RT in a rocking platform. To stain the nuclei, organoids were then incubated with DAPI at a 1:2500 dilution in 1X TBS. To mount the stained organoids for microscopy visualization, the organoid was placed on a microscope glass slide and the excess of DAPI solution was removed before adding on top of one or two drops of RapiClear (SunJin Lab, RC147001) as mounting medium. Then, a round coverslip (Thermo Fisher Scientific, CBAD00120RAC20MNZ#0) was carefully placed to cover the sample, and the excess of mounting medium was dried before covering the borders of the coverslips with nail polish to fix them to the slides. Samples were visualized using a Super-resolution Inverted Confocal Microscope Zeiss LSM 880-Airyscan Elyra PS.1 and Confocal Microscope Zeiss LSM 800.

3.8.3. Images quantification

Quantification of sarcomere length based on ASA staining in IF images was performed using Image J software and custom python code. Briefly, segments were manually annotated perpendicular to at least 4 z-disc lines depicted by ASA⁺ staining. Traced annotations allowed to measure profiles of fluorescence intensity across distances between adjacent ASA⁺ filaments and successively, the mean sarcomere length was determined by averaging the distances between intensity peaks. Segments were annotated in a blind approach and with enough relative distance enabling the measure of sarcomeres from at least 20 different cells by condition.

Quantification of fluorescent areas corresponding to the staining of determined markers in IF images was performed with Image J software. Briefly, binary masks from sum projections of each staining were generated using the Auto-Threshold tool from ImageJ. The thresholded area was then quantified with the Measure tool from ImageJ. In the analysis of the quantified data, the values of areas for COLLAGEN IV, LAMININ and FIBRONECTIN stainings were normalized to values of areas for DAPI. Quantification results were represented with graphs generated using GraphPad Prism version 9.0.0 software.

3.8.4. Primary and secondary antibodies for ICC

Primary and secondary antibodies were diluted according to manufacturers' indications or empirical optimization, as indicated in the following table:

Table 14: Primary and Secondary Antibodies for ICC

Primary Antibodies	Host	Dilution	Cat#
Oct-4	Mouse	1:25	sc-5279
Nanog	Goat	1:25	AF1997
Sox2	Rabbit	1:100	PA1-16968
GFP	Chicken	1:250	GFP-1020
RFP	Rabbit	1:400	ab34771
Alpha-Sarcomeric Actinin	Mouse	1:500	MA1-22863
Vimentin	Rabbit	1:200	ab92547
Troponin T	Mouse	1:500	MA512960
MYH6	Mouse	1:100	GTX20015
CD31	Rabbit	1:50	ab28364
Connexin 43	Rabbit	1:500	ab11370
Collagen I	Rabbit	1:50	ab34710
Collagen IV	Goat	1:50	ab769
Fibronectin	Rabbit	1:100	ab2413
Laminin	Rabbit	1:50	L9393
PODXL	Goat	1:100	BAF1658
Alpha-Galactosidase	Mouse	1:10	M86
LTL	-	1:200	B-1325
Secondary Antibodies	Host	Dilution	Reference
anti-mouse 555	Donkey	1:200	A31570
anti-mouse 488	Donkey	1:200	A21202
anti-mouse 647	Donkey	1:200	A-31571
anti-goat 647	Donkey	1:100	JAC-705-605-147
anti-rabbit 555	Donkey	1:200	A11015
anti-rabbit 647	Donkey	1:200	A-31573
anti-rabbit 488	Donkey	1:200	A21206
anti-chicken 488	Donkey	1:100	703-225-155
Dylight 649 Streptavidin	-	1:40	SA5649

Note: 10X TBS for ICC was prepared by dissolving 24 g TRIZMA base (Sigma, T6791) and 88 g NaCl (Sigma, S7653) in 800 mL MiliQ H₂O. Then pH was adjusted to 7.6 with HCl (37% HCl, Panreac, 211020-1611), and MiliQ H₂O was added to complete the volume to 1L.

3.9. Oxygen consumption rate (OCR)

The measurement of OCR cardiac organoids was performed using XFe24 extracellular flux analyzer (Seahorse Bioscience) as we previously described in kidney organoids (230). Briefly, one organoid is plated in each well of a Sea horse cell culture microplate. Uncoupled and maximum OCR were assayed with oligomycin (10 mM)

and FCCP (2 mM). To inhibit complex I- and III-dependent respiration, rotenone (5 mM) and antimycin A (15 mM) were used, respectively. OCR represents the oxygen tension and acidification of the medium as a function of time (pmol min⁻¹). OCR was normalized to protein quantity in each well.

3.10. Single-cell RNA sequencing

Single-cell RNA sequencing was performed in collaboration with “Centro de Investigacion Medica Aplicada” (CIMA) in Pamplona. Cardiac-like organoids were collected and washed twice with PBS, and further dissociated into a single cell suspension by treating them with Accumax for 15 min at 37°C followed by Trypsin-EDTA 0.25% (wt/vol) trypsin (25300-054, Life Technologies) for additional 15 min at 37°C. The reaction was deactivated by adding 10% FBS. The cell suspension was then passed through a 40µm cell strainer. After centrifugation at 1,000 rpm for 5 minutes cell numbers and viability were analyzed using Countess Automated Cell Counter. Then cell suspensions were loaded onto a well of a 10x Chromium Single Cell instrument (10x Genomics). Barcoding and cDNA synthesis were performed according to the manufacturer's instructions. Qualitative analysis was performed using the Agilent Bioanalyzer High Sensitivity assay. The cDNA libraries were constructed using the 10x Chromium™ Single cell 3' Library Kit according to the manufacturer's original protocol. Libraries were sequenced on an Illumina HiSeq or NextSeq 2x150 paired-end kits using the following read length: 28bp Read1 for cell barcode and UMI, 8bp I7 index for sample index and 94bp Read2. The median number of unique molecular identifiers (UMIs) per cell was between 2,300 and 4,600, with a median of 1,300-2,200 genes detected per condition.

3.10.1. scRNA Seq analysis

ScRNAseq analysis was performed by Dr. Asier Ullate under the supervision of Dr. Felipe Prosper from Clínica CIMA. The computational analysis of the resulting UMI count matrices was performed using the R package Seurat. Poor quality cells were removed. For the latter, the upper thresholds were obtained from visual inspection of the UMI and detected genes distribution. Each dataset was subjected to normalization,

identification of highly variable features and scaling using the *SCTransform* function, regressing out for the number of detected genes, the % of mitochondrial UMIs and cell cycle. Clustering was performed by setting the resolution parameter to 0.6 in both cases. Dimensional reduction for data visualization was done using the *RunUMAP* function. Cell markers in each cluster were identified using the *FindConservedMarkers* and *FindAllMarkers* functions in the non-integrated counts by using the Wilcoxon Rank Sum test. Genes with p-value < 0.05 (adjusted by Bonferroni's correction) were retained. Clusters were labelled by comparing the expression of the identified markers with publicly available databases. Differential expression analysis to identify changes across conditions were also performed using the Wilcoxon test, keeping as up-regulated genes those present in a fraction of 0.1 of either population, with a log fold-change greater than 0.1 and with an adjusted p-value < 0.05. To perform over-representation analysis the R package *enrichR* was used with the database "MSigDB_Hallmark_2020". Gene set enrichment analysis (GSEA) was done with the *fgsea* R package, considering the hallmark gene sets from MSigDB.

3.11. Calcium transient analysis

Optical mapping analysis were performed at the Translational Cardiology Laboratory of the Hospital General Universitario Gregorio Marañón (HGUM) in Madrid by Antonio Sanchez Salgado under the supervision of Dr. María Eugenia Fernández and Gonzalo Rios Muñoz Leganes (231). Cardiac- like organoids were subjected to dye loading for approximately 30 min before being washed out to initiate the mapping process. Following a standardized protocol, each organoid underwent a 5-sec acquisition at increasing frequencies. This involved capturing videos at the basal stage and stimulating the cells at 1Hz, 2Hz, 3Hz, and 5Hz. Subsequently, a final basal acquisition was recorded at a higher macro zoom level. Once all cells were analyzed, flecainide was administered for 5 minutes, followed by 2 extra basal acquisitions. Ratiometric fluorescent dye Fura-2F-AM (Thermo Fisher Scientific, USA) is used to measure intracellular calcium levels. The excitation wavelengths for Fura- 2F-AM are 340 nm and 380 nm. The ratio of the fluorescence signals is influenced by the concentration of Ca²⁺. In the presence of calcium, Fura- 2F-AM emits a red fluorescence, whereas, in the absence of calcium, it emits a green fluorescence. A vial containing 5 mg of Fura-2F-AM dye is dissolved in 8 mL of DMSO to prepare aliquots of 20 µL. Each 20

μL aliquot was dissolved in 3 mL of Tyrode solution (for one p60 or two p35s), resulting in a final concentration of 4 μM in the well once mixed with Tyrode (12 $\mu\text{g}/3\text{ mL}$ Tyrode). To excite the Fura-2F-AM dye, two UV light-emitting diodes (LEDs) were utilized. The first LED was a UVMAX335-FX-50 model (Roithner LaserTechnik, Vienna, Austria) with a peak power output of 15mW and a peak wavelength of 340 nm. For collimation, the light emitted by Ex1 was passed through a UV-fused silica bi-convex lens (LB4879; Thorlabs, USA). The second LED was an NCSU034B model (Nichia, Tokushima, Japan) with a higher peak power output of 540mW and a peak wavelength of 385 nm. To collimate the light emitted by Ex2, a planoconvex lens (LA1951; Thorlabs, USA) made of UV fused silica was used. These arrangements ensured efficient and controlled excitation of the Fura-2F-AM dye for subsequent imaging and analysis. The camera used as a detector is an Evolve 128 (Teledyne Photometrics, Arizona, USA). It is a back-illuminated, electron-multiplying CCD (EMCCD) camera. It has a 128x128 pixel array with a pixel size of 10 μm , which provides a high spatial resolution. It counts with a liquid cooling system, reaching up to -85°C , to reduce dark current noise. The camera is controlled using custom software and using the drivers available at the manufacturer's website. The camera is coupled to a macroscopic objective MVX10 (Olympus, Japan) to zoom in on cells. The objective has a magnification of up to 6.3x. The equivalence table is used to calibrate the acquisition and calculate the size of the cells. The table provides a mapping between the size of the cells in the image and their actual size in micrometers. This information is essential for accurate measurements of cell size and morphology. Additionally, a specialized electrical stimulator is integrated into the experimental setup. A MATLAB code (OM Analyzer) was used to perform image recording and processing.

3.12. Decellularization and dECM-derived hydrogel

3.12.1. Decellularization of porcine and human kidney organs

Porcine kidneys were harvested from adult healthy pigs under general anesthesia. The experimental procedure was approved by the ethics committee of Hospital Clinic de Barcelona. Renal tissue from partial nephrectomies were used in this study. The experimental procedure was approved by the ethics committee of Hospital Clinic de

Barcelona. Samples were preserved at -80 °C until further use. For decellularization, frozen tissues were thawed on ice. The renal cortex was separated from the medulla manually. Specific tissue samples were then minced into small pieces that were placed in a sterile glass recipient and rinsed several times with PBS supplemented with penicillin/streptomycin and milliQ H₂O while maintained under constant agitation to remove the remaining blood and generate hypotonic cell lysis. The washes were performed until the solution had a transparent appearance. Next, renal cortex slices were decellularized by immersion into 1% sodium dodecyl sulfate (SDS; Bio-Rad, Sigma, 161-0301) in deionized water while shaking for 6 days with solution changes every 2h during the first 2 days and twice a day thereafter. Finally, samples were rinsed thoroughly with PBS containing antibiotics for 1 additional day, and with deionized water for another 5 days.

3.12.2. Decellularization of porcine heart organ.

Porcine hearts were harvested from adult healthy pigs under general anaesthesia. The experimental procedure was approved by the ethics committee of Hospital Clinic de Barcelona. Samples were preserved at -80 °C until further use. For decellularization, frozen heart tissues were thawed on ice. Ventricles were separated from the atriums manually and tissue samples were then minced into 1 cm² pieces that were placed in a sterile glass recipient and rinsed several times with milliQ H₂O while maintained under constant agitation to remove the remaining blood. Then, they were washed O.N. with PBS supplemented with penicillin-streptomycin. Next, ventricle pieces were decellularized by immersion into 2% SDS in milliQ H₂O while shaking for 3 days with solution changes twice a day, followed by immersion into 1% SDS for another 7 days with solution changes once a day. Finally, samples were rinsed thoroughly with milliQ H₂O for 7 days.

3.12.3. Preparation of kidney and heart dECM-derived hydrogels

Decellularized renal and cardiac slices were placed at -80°C for 24h and lyophilized for 48h. The lyophilized tissue was crushed into powder using IKA tube Mill 100 machine. The required amount of dECM powder was weighted and digested in a

solution of 0.01N hydrochloric acid (ITW Reagents, 131020) with 6 mg of pepsin (Sigma, P7012-1G) for 60 mg of dECM for 72h under constant agitation. Following digestion with pepsin, the dECM solution was supplemented with 10X concentrated PBS or culture media to maintain the osmotic pressure. The dECM pre-gel was stored at -20°C until further use. For immediate use or when defrost, pH was adjusted to around 7 using 0.1 M sodium hydroxide (Sigma, S2770) while keeping the solution in ice. For the DNA quantification, genomic DNA was isolated from native kidney and heart and kidney and heart dECM samples using the DNeasy Blood & Tissue Kit following manufacturer's instructions. DNA samples were then quantified by spectrophotometry (Nanodrop).

3.13. Combination of hPSC-derived organoids with dECM-derived hydrogels

3.13.1. Encapsulation of hPSCs-kidney progenitor cells with renal dECM-derived hydrogels

The bioinks formulations based on gelatin and fibrin were prepared as indicated below prior preparation of stock solutions of the different components. A stock of 15 wt/v% gelatin (type A from porcine skin, G1890, Sigma) was prepared by dissolving the gelatin in sodium chloride solution (9 wt/v% NaCl, 10 mM Hepes) under constant stirring at 70° C for 8 h. Once dissolved the gelatin stock solution was filtered using a 0,22 µm filter and stored at 4° C. The fibrinogen (F8630, Sigma) and transglutaminase (BDF PROBINDR) were dissolved in sodium chloride at a concentration of 50 mg/mL and 60 mg/mL, respectively, and kept at 37 °C for 30 min to allow complete dissolution. A CaCl₂ stock solution (250 mM) was prepared by dissolving CaCl₂ powder (C7902, Sigma) in sodium chloride solution. To prepare a stock solution of thrombin, lyophilized thrombin (T4648, Sigma) was reconstituted at 100 U/mL using sterile water and 0,1 wt/v % bovine serum albumin (A8806, Sigma) and stored at -20 °C.

The bioink was prepared just before the encapsulation with hPSCs-kidney progenitor cells (*D4*, see section 3.4) and the solutions mixed together at 37 °C achieving a final concentration of 5 mg/mL fibrinogen, 5% gelatin, 2,5 mM CaCl₂ and 0,1% transglutaminase with or without 15 wt/v% dECM and a final density ranging of progenitor cells from 15 to 25 million cells/mL in 200 ng/ml FGF9 and 1 µg/mL heparin in advanced RPMI 1640 basal medium.

Drops of hydrogel with hPSCs-kidney progenitor cells were seeded in trans-well inserts (Sarstedt, 83.3931.041) and it was maintained for 10 min in ice and then cross-linked with 10 U/mL thrombin resuspended in sodium chloride solution for 30 min at RT. Finally, it was washed once with sodium chloride solution and left in the Advanced RPMI media with factors until *D11*. From *D12* onwards, the media change was made every 48 h with differentiation basal medium without growth factors until *D20*.

3.13.2. Combination of hPSCs-derived cardiac-like cells with cardiac dECM-derived hydrogels

2D cell culture condition. In monolayer differentiation, beating cardiomyocytes from *D12* until *D35* were grown in cardiac media (RPMI/B27+insulin) supplemented with/without cardiac dECM-derived hydrogel (dilution 1:20).

Floating condition. For the 3D-floating differentiation, beating cardiomyocyte monolayers at *D12* of differentiation (see section 3.3.1) were dissociated in single cells using Accumax for 3 min at 37°C. Then, single- cells were collected and based on viability and cell number, cells were resuspended at 5×10^4 (50K) cells in RPMI/B27+insulin medium supplemented with 5uM ROCK inhibitor. To generate the organoids, 100 μ L of the adjusted cell suspension were pipetted in each well of a conical-bottom 96-well plate, which was afterwards centrifuged for 3 min at 300 *g*. The 96-well plate was then maintained in an incubator at 37 °C with 5% CO₂ for 24 h without medium change to allow cells to self-aggregate into a free-floating spheroid. After 24 h, RPMI/B27+insulin medium was replaced with fresh B27+insulin media with/without cardiac dECM-derived hydrogel (dilution 1:20) and 50K cardioids were maintained in this medium until *D35* with medium change every 2 days.

Insert condition. Beating cardiomyocyte monolayers at *D12* of differentiation (see section 3.3.1) were dissociated in single cells using Accumax for 3 min at 37°C. Cell suspension was plated into AggreWell800 microwell culture plates (Stem Cell Technologies, 34811) at cell densities of $1,5 \times 10^6$ cells per well in 1,5 mL of RPMI/B27 medium supplemented with 5 μ M ROCK inhibitor. Plates were then centrifuged at 2000 rpm for 3 min to induce 5000 (5K) micro-organoids formation into aggrewell and left for 24h to form cell aggregates. After 24 h, 5K cardioids were seeded on either collagen without/with dECM coating covered trans-well inserts and were maintained

in cell culture until *D35* of differentiation with media change every 2 days with/without cardiac dECM-derived hydrogel (dilution 1:20) in cardiac media (RPMI/B27+insulin). The bioinks formulation for the collagen coating was prepared as indicated below prior preparation of the different components' solutions. A stock of collagen (CellSystems, 5005) was prepared by mixing 10X PBS and cardiac media (RPMI/B27+insulin) and collagen at a concentration of 2 mg/mL and neutralized bringing the pH to 7.4. Once prepared the collagen stock, 2 different solutions were prepared: collagen without cardiac dECM-derived hydrogel, diluting the solution at 1.5 mg/mL with cardiac media and collagen with cardiac dECM-derived hydrogel, diluting the stock solution with 1 mg/mL of cardiac dECM-derived hydrogel at a final concentration of 1.5 mg/mL.

RESULTS

4. RESULTS

Chapter I: Generation of cardiac reporter cell lines for the assessment of cardiac differentiation lineage in hPSCs

4.1. Generation of cardiac reporter cell lines using TALEN and CRISPR/Cas9 genome editing tools

The generation of reporter cell lines specific for cardiac genes by genome editing are valuable tools for monitoring *in vitro* cardiac development (202). At the same time, such developments allow to improve differentiation protocols, as well as isolating and characterizing specific hPSC-derived cardiomyocytes populations (56,207,232,233). MYH6 is an essential transcription factor for cardiac muscle contraction expressed at early and late stages during cardiac differentiation in humans (88–90). Due to its fidelity in terms of expression during all the onset of cardiac differentiation, MYH6 has been the gene of choice to monitor early and late stages of cardiomyocyte differentiation from hPSCs (210,211). In fact, in 2016 the host laboratory generated a hESC MYH6 reporter cell line by means of TALEN. By targeting the first exon of MYH6 with mCherry fluorescent reporter we demonstrated that is possible to preserve the regulatory sequences near the native ATG start codon for the translation of the gene. This approach enabled the definition of a robust protocol for the generation of cardiac-like cells (CLCs) from several hPSC lines, including hESCs and hiPSCs (56). Moreover, other transcription factors can serve as useful tools for monitoring cardiac processes. In this regard, taking advantages of the iC2 platform recently developed in the host laboratory (148,229), we have generated reporter cell lines for MYH6 as well as MYL2, a sarcomeric protein playing an essential role in early embryonic cardiac development and one of the earliest markers of ventricular specification. We have also generated reporter cell lines for SIRPA, a cell surface marker for emerging cardiovascular lineages previously targeted in hPSCs (234). The abovementioned iC2 is an optimization of iCRISPR, a cellular platform originally developed in 2014 by Gonzalez et al. (199). The original platform already enabled the generation of stable and inducible knock-out and knock-in hPSCs controlled by a Cas9 expression via doxycycline (DOX) treatment, though further applications related to transgenesis was limited because of antibiotic resistance and biallelic modification of the AAVS1 *locus*.

Overall, iC2 allows rapid and highly efficient generation of constitutive and inducible knock-out, knock-in and reporter hPSC lines. Here we have exploited the iC2 platform for the generation of different hPSC reporter lines used in this thesis.

4.1.1. Generation of MYH6, MYL2 and SIRPA cardiac reporter cell lines exploiting the iC2 platform

We have generated different reporter cell lines in both female and male iC2 backgrounds. In one hand, MYH6, MYL2 and SIRPA reporter cell lines have been generated in the iC2 platform previously described in the laboratory. mOrange fluorescent marker was integrated at the end of *MYH6*, *MYL2* and *SIRPA* coding sequences making use of the iC2 line 2 (female hPSCs). On the other hand, the GFP fluorescent marker was integrated at the end of *MYH6* coding sequence making use of the iC2 line 1 (male hPSCs).

The procedure used to generate all the different lines is described here. The first step consisted into the cloning of the specific donor plasmid (pDonor) and pRR-puro plasmid for each gene of our interest (GOI). The pDonor was cloned with the sequence for the expression of the fluorescent protein of choice flanked by 2 homology arms (HA), corresponding to the targeted sequence of the GOI for its correct integration through HDR repair, an HA epitope, allowing the eventually selection and purification of the protein of interest and a T2A peptide. The T2A self-cleaving protein induces ribosome skipping bringing to the expression of GOI and fluorescent markers as 2 separate proteins by avoiding the formation of fusion proteins (235) (Fig. 7A). The pRR-puro plasmid was designed with a N-terminal puromycin resistance sequence prematurely terminated followed by the PAM sequence specific for the sgRNAs designed for each GOI and a full-length coding sequence of the puromycin resistance gene (236) (Fig. 7B). Successively, 4 different sgRNAs were designed for each GOI taking advantage of the bioinformatic web-tool <https://eu.idtdna.com> that allows the choice of the region in which the DSB induced by sgRNAs must occur. In contrast to our first approach making use of TALEN mediated genome editing to target the *MYH6* locus with the mCherry reporter, here we have selected a region around the stop codon of the last exon of each GOI to ensure full transcription and conversely protein transduction (Fig. 7A). The 4 sgRNAs were tested by transfection in iC2 hPSC lines

followed by sequencing analysis from transfected pooled cells. Briefly, to select the sgRNAs with higher editing efficiency, we first analyzed the percentage of insertions or deletions of one or several nucleotides within the target sequences (Indel percentage) after their transfection in hPSCs. The indel percentage was analyzed by TIDE web-tool, which compares the edited samples against a control by analyzing their sequences obtained by Sanger sequencing after PCR amplification of a fragment enclosing the designed editing site. CRISPR/Cas9 induced by nucleofection allows the introduction of the fluorescent protein provided by the pDonor by HDR in a desired *locus* driven by sgRNAs. The strategy for the generation of reporter cell lines consisted of a co-nucleofection in DOX treated iC2 hPSCs lines 1 and 2 of the above-cited components: pDonor, pRR-puro plasmid and sgRNAs. After the co-nucleofection, cells were plated as single cells and cultivated for a couple of days before starting antibiotic treatment for the following 3 days. After 3 days, puromycin-resistant cells colonies start to grow until reaching an optimal confluence for the picking. To confirm the positive clones, picked colonies were screened by PCR analysis (Fig. 7C).

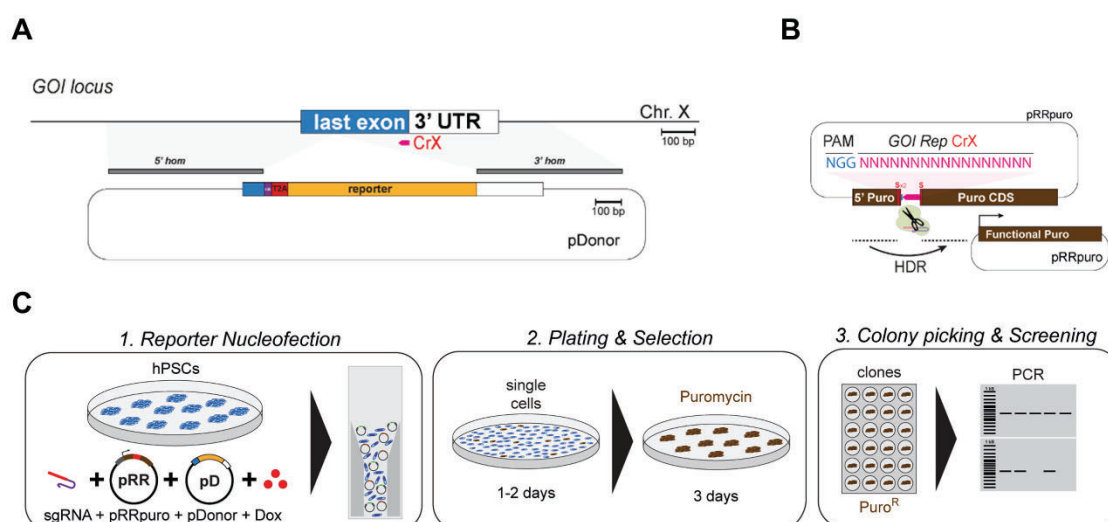


Fig. 7. Gene editing strategy for the generation of hPSC reporter using iCRISPR2. **A)** Strategy for the pDonor design. Representation of the sequence of the GOI *locus*. The sgRNAs were designed around the stop codon of the GOI. The blue box represents the last exon of the GOI. The white box represents the 3' UTR sequence. The pink arrow represents the selected sgRNA. **B)** Strategy for the pRR-puro design. Representation of the pRR-puro plasmid used as a selector of HDR-mediated insertions. The pRR-puro plasmid contains the sgRNA protospacer sequence (pink) together with the PAM (blue) flanked by a prematurely terminated 5' puromycin (puro)-resistance sequence (short brown box) and a complete puro-resistance coding sequence (CDS) (large brown box) with a stop codon instead of a start codon (red lines). **C)** Workflow scheme for the generation of hPSC reporter cell line. First, sgRNAs were co-nucleofected with pRRpuro and donor plasmids in DOX treated hPSC cell lines. Cells were plated as single cells and after 1-2 days were treated with antibiotics during 3 days. Puromycin-resistant colonies were picked and gDNA extracted for PCR colony screening.

Here, the strategy for the pDonor and pRR-puro design for MYH6, MYL2 and SIRPA. For all the genetically engineered lines, the reporter gene insertion was desired at the end of the coding sequence, to ensure the full and correct protein expression. Human *MYH6* gene is located on chromosome 14 and the selected sgRNA (Cr3) was designed on exon 36 around the stop codon. The pDonor was designed to allow the insertion of a GFP and/or orange fluorescent protein (mOrange) downstream *MYH6* in iC2 hPSCs lines (Fig. 8A and 8B). On the other hand, *MYL2* is located on chromosome 12, and the chosen sgRNA (Cr1) precisely cleaves at the end of the exon 7 (Fig. 8C). Furthermore, the *SIRPA* gene is located on chromosome 20, and the sgRNA (Cr4) was specifically designed to target the exon 8 (Fig. 8D).

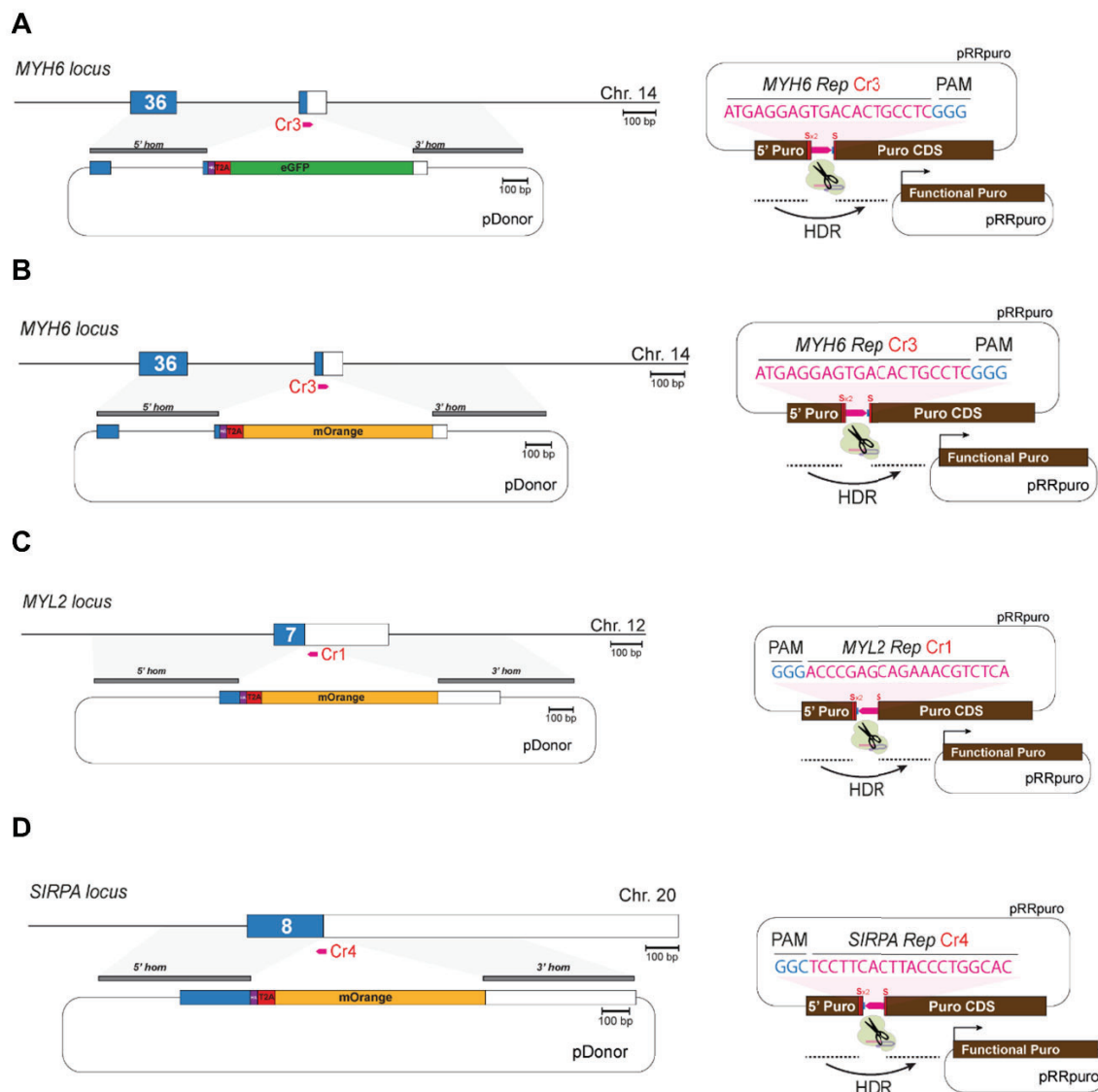


Fig. 8. Strategy design for the pDonor and pRR-puro plasmids. A) Representation of sequences of the *MYH6* locus where sgRNAs were designed (Cr3) around the last exon 36 (blue box). The pDonor plasmid contains the end of exon 36 of *MYH6* gene without the stop codon, which will serve as 5' HA, followed by the sequence for the expression of the T2A peptide and the green fluorescent protein (GFP). The GFP sequence is followed by the 3' UTR sequence of the *MYH6* gene (white box) which will serve as 3' HA. The pRR-puro plasmid contains the *MYH6* Rep Cr3 sgRNA sequence together with the corresponding PAM flanked by a prematurely terminated 5' puromycin (puro)- resistance sequence and a complete puro-resistance coding sequence (CDS) with a stop codon instead of a start codon. **B)** Analogous to figure A with a mOrange fluorescence protein. **C)** and **D)** Analogous to figure B for *MYL2* and *SIRPA* locus.

After the co-nucleofection and the antibiotic treatment, the strategy for identifying the positive targeted clones was based on PCR screening. Primers were designed to amplify specific sequences of the 2 possible alleles: wild type (WT) or reporter integration. A PCR performed with a forward (5H-F) and reverse primer (3H-R) designed outside the insertion site allow the PCR amplification of a specific WT sequence when the insertion did not occur. On the other hand, 5H-F and 3H-R primers coupled with a reverse and forward primers annealed inside the fluorescence, can allow the identification of the correct integration of the fluorescence protein provided by the donor plasmid (Fig. 9A).

First, a preliminary screening was performed confirming the correct integration of the donor plasmid by performing PCR on one of 5' or 3' Homology Arms. In this case, we performed 3H PCR, and in green (GFP reporter) and orange (mOrange reporters) the positive clones (Fig. 9B). Then, a second PCR for both HA was performed and only the positive clones for both HA underwent to a third PCR round to identify the presence of 1 or 2 targeted alleles by using 5H-F with 3H-R primers (full-length- FL PCR). In this way, we distinguished the generation of heterozygous or homozygous clones. The homozygous clones were selected as candidates for the further cardiac differentiation experiments (Fig. 9C). The lines selected for further analysis were hPSC MYH6-GFP #C4, hPSC MYH6-mOrange #G12, hPSC MYL2-mOrange #G11, and hPSC SIRPA-mOrange #A8.

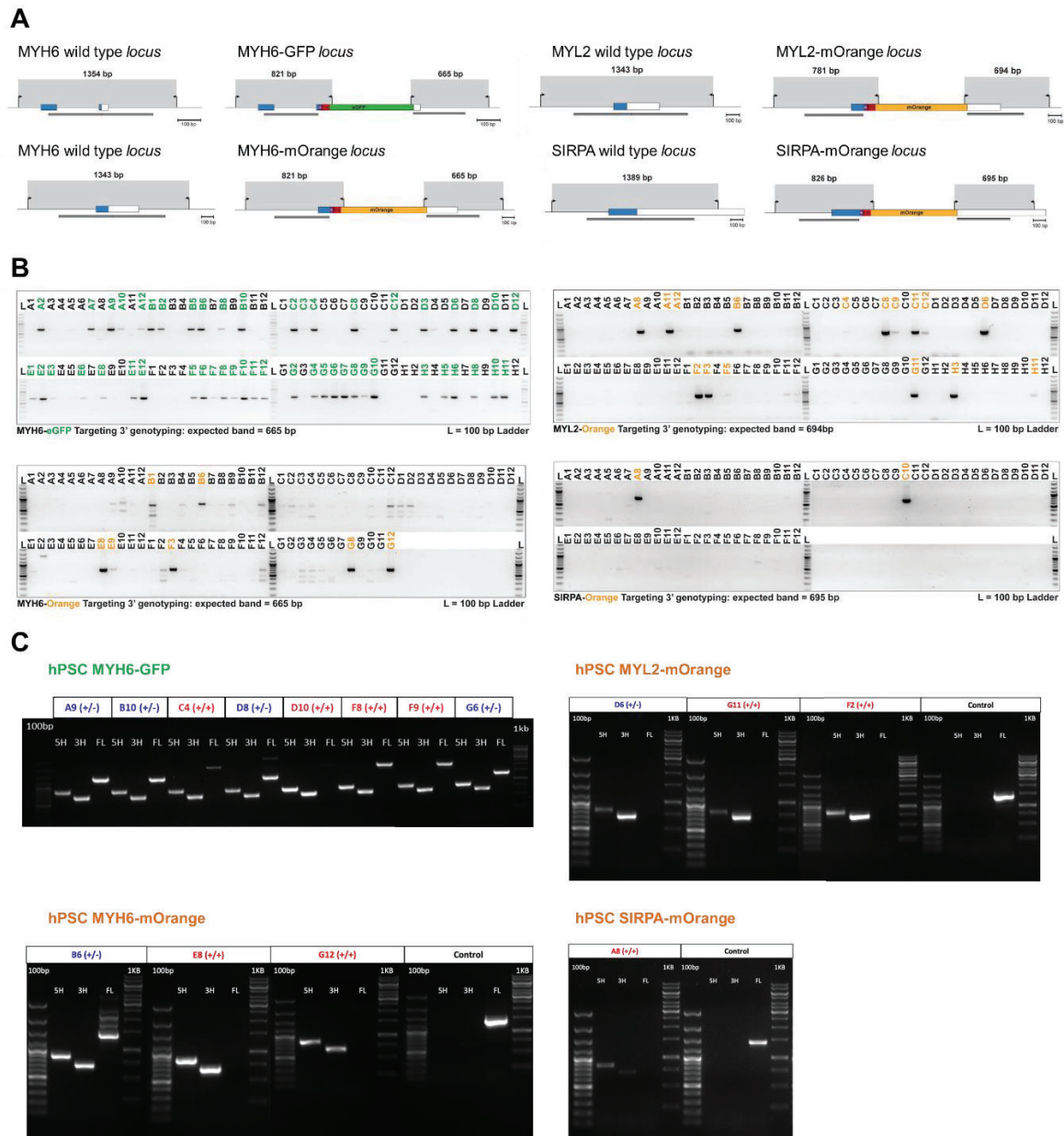


Fig. 9. PCR genotyping of reporter cell lines. A) Representation of the PCR genotyping strategy to identify wild type (WT) and targeted alleles. The WT PCR primers were designed within the 5' and 3' HA of the *MYH6*, *MYL2* and *SIRPA* loci. The PCR primer pairs to identify the 5' and 3' ends of the reporter integrated sequence were designed outside the 5' and 3' HA and within the fluorescence sequence. **B)** Colony screening by PCR analysis using the 3H primers. **C)** Final PCR for each reporter performing 5H, 3H and full-length (FL) PCRs showing homozygous (red) and heterozygous (blue) clones.

4.1.2. Characterization of hPSC reporters for pluripotent-related features including quantitative PCR analysis and immunofluorescence and confocal microscopy analysis

It has been widely reported that hPSC are sensitive to culture pressure and growth expansion (237). In this regard, phenotypic features related to pluripotency were analyzed through quantitative PCR and immunofluorescence analysis. The mRNA expression levels of established pluripotency gene markers including *OCT4*, *NANOG*, *REX1*, *DNMT3b* and *CRIPTO* were analyzed by qPCR. Non-edited iC2 hPSC lines and all the relative hPSC reporter lines showed similar levels of pluripotency markers (Fig. 10A). These data were confirmed through immunofluorescence and successive confocal microscopy analysis showing OCT4, NANOG and SOX2 expression in hPSC reporter cell lines and absence of reporter markers, as expected (Fig. 10B).

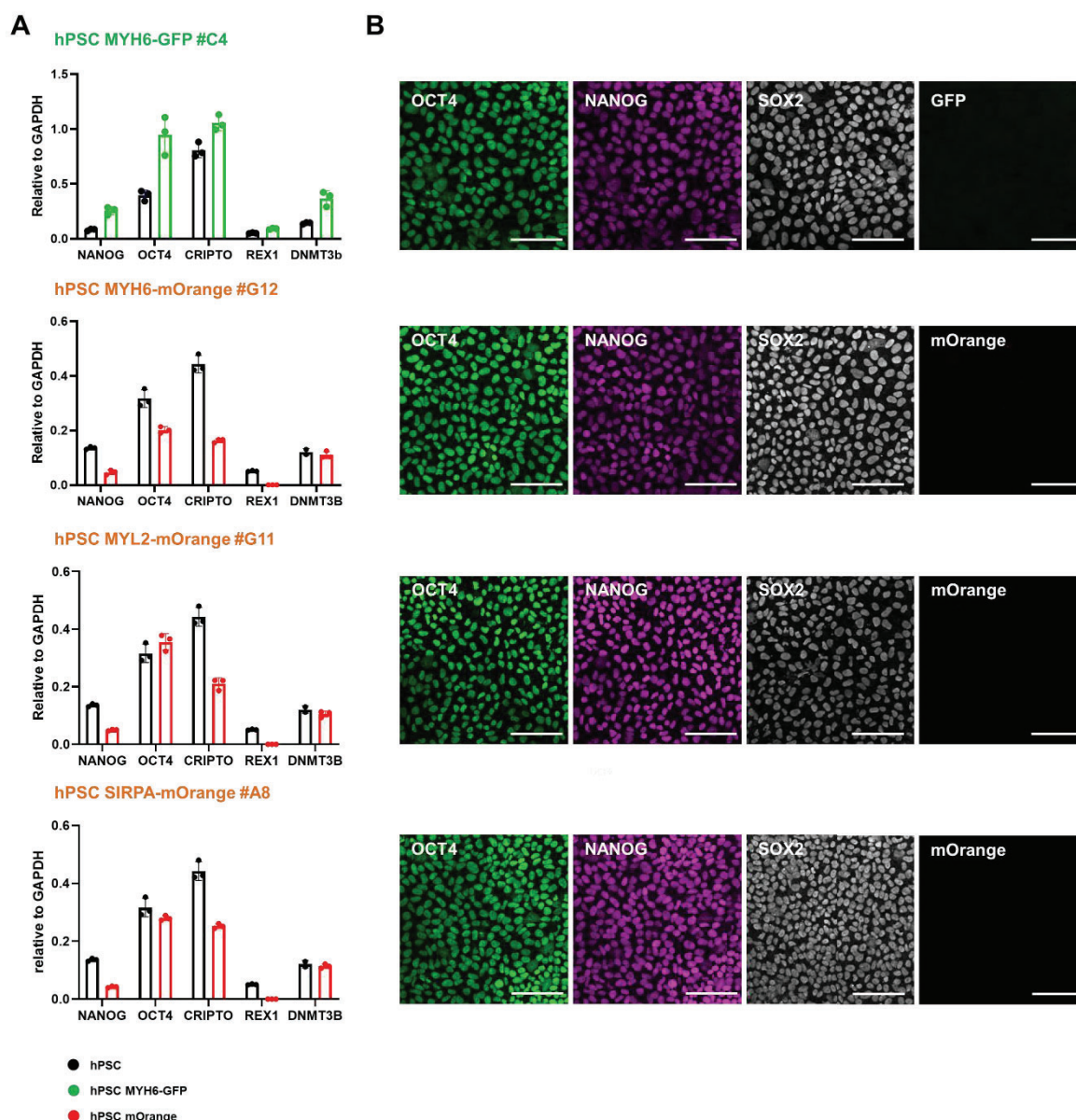


Fig. 10. Characterization of hPSC reporters for pluripotent-related features. **A)** Representative qPCR analysis of the pluripotency markers *NANOG*, *OCT4*, *CRIPTO*, *REX1* and *DNMT3b* in iC2 hPSC lines (black) compared with reporter cell lines generated in the same cell MYH6-GFP #C4 (green), MYH6-mOrange #G12 (red), MYL2-mOrange #G11 (red) and SIRPA-mOrange #A8 (red) cell lines. Data are mean $\pm$ SD. $n = 1$ independent experiment with 3 technical replicates. **B)** Representative images of the immunofluorescence analysis performed to detect the expression of the pluripotency markers OCT4 (green), NANOG (magenta) and SOX2 (grey) in hPSC MYH6-GFP #C4, MYH6-mOrange #G12, MYL2-mOrange #G11 and SIRPA-mOrange #A8 cell lines. GFP and mOrange detection confirmed the absence of MYH6, MYL2 and SIRPA expression at the pluripotent stage. Scale bar 100 μ m.

Aside the characterization of pluripotency markers by qPCR and IF analysis, we further evaluated the capability of genetically engineered hPSC clones to differentiate towards the 3 germ layers of the embryo including: ectoderm, endoderm, and mesoderm lineages. For this purpose, colonies of each cell line were detached in

single cells and re-aggregated by spin centrifugation to force embryoid bodies (EBs) formation. After 3 days, EBs were seeded on gelatin-coated slide flasks and exposed to specific culture conditions for the generation of mesoderm, endoderm, and ectoderm derivatives making use of procedures previously established in the host laboratory (238). After 2 weeks, immunocytochemistry was performed to detect the expression of mesodermal (VMT and SMA), neuronal (PAX6 and TUJ1) and endodermal markers (AFP, FOXA2) (Fig. 11). Even if by the time for the preparation of the thesis, endodermal differentiation results were pending for hPSC MYH6-mOrange #G12 and hPSC SIRPA-mOrange #A8. Our results suggest that pluripotency is preserved after genetic engineering of iC2 hPSC lines.

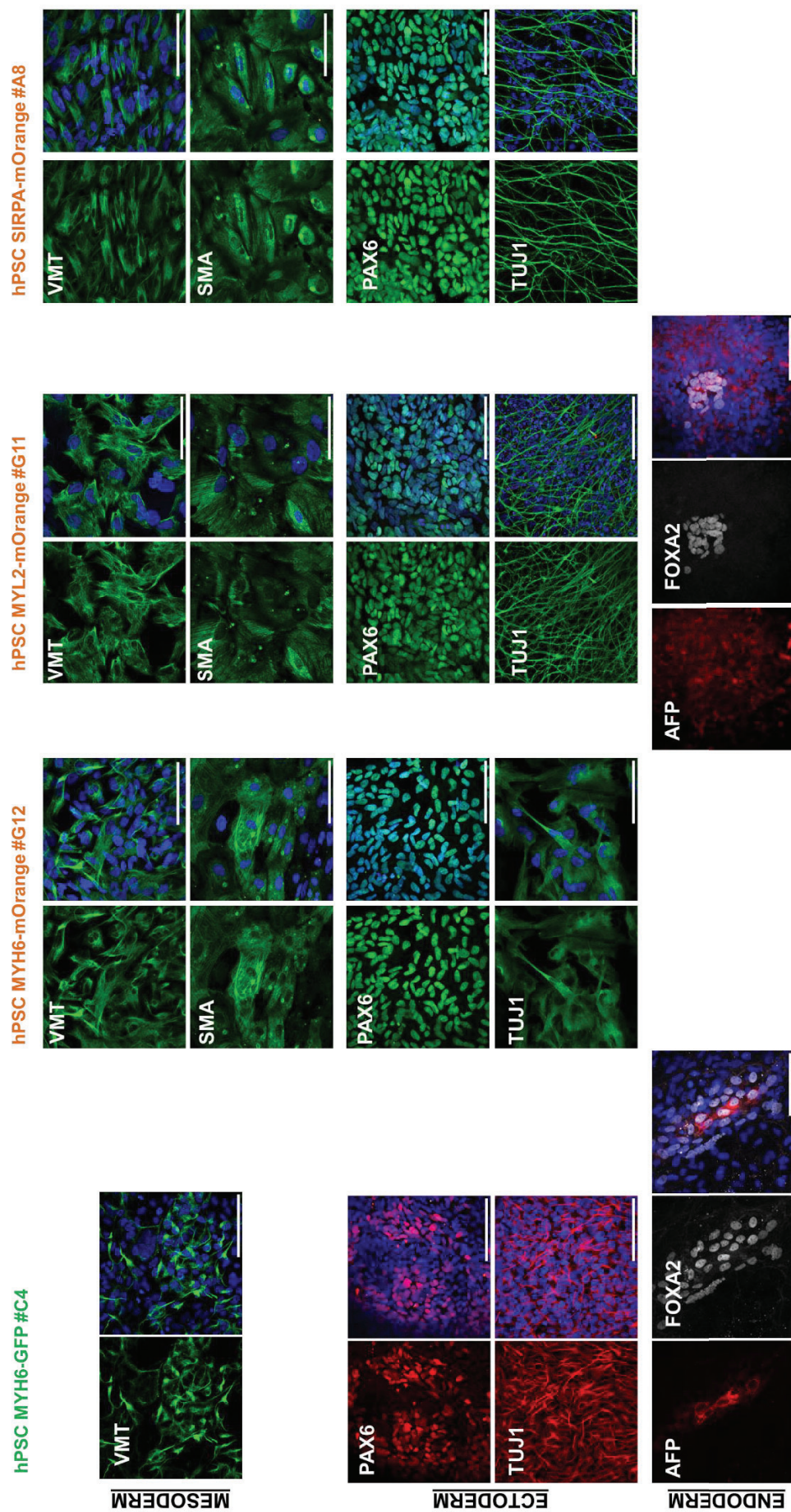


Fig. 11. Characterization of hPSC reporters *in vitro* differentiation potential. Representative images of the immunofluorescence analysis performed in hPSC MYH6-GFP #C4, hPSC MYH6-mOrange #G12, hPSC MYL2-mOrange #G11 and hPSC SIRPA-mOrange #A8 cell lines subsequently EBs formation and differentiation toward the 3 germ layers. Mesoderm layer is characterized by the expression of VIMENTIN (VMT, green) and smooth muscle actin (SMA, green), ectodermal by PAX6 (red/green) and TUJ1 (red/green) and the endodermal by alpha-fetoprotein (AFP, red) and FOXA2 (grey). Scale bar 100 μ m. Experiments for the characterization of the *in vitro* differentiation potential of the lines indicated here were performed independently twice.

4.2. Differentiation and characterization of hPSCs cardiac reporter lines towards cardiac-like cells (CLCs) in 2D cell culture conditions

To further assess the utility of the reporter cell lines generated in this thesis, all the engineered cell lines were tested for their capacity to differentiate towards cardiac-like cells and their fidelity to monitor the endogenous expression of the cardiac marker of choice [at both transcriptomic and protein level(s)]. First, hPSC reporter lines were differentiated in 2D using a differentiation protocol already established in the host laboratory (56). Successively, starting from the 2D we have generated self-aggregates in 3D composed of multiple cell types enabling us to effectively evaluate their potential as highly valuable human disease model.

4.2.1. Establishment of cell culture conditions supporting the derivation of CLCs from hPSCs cardiac reporter lines in 2D

To explore the potential use of the engineered lines generated in this thesis, we first made use of a 2-step differentiation procedure previously described in the host laboratory to generate CLCs from hPSCs (56). In accordance with the protocol described in material and methods (section 3.3.1), this approach makes use of cell culture conditions in which undifferentiated hPSCs are grown as a monolayer and further exposed to biochemical factors activating Wnt endogenous pathway upon which the same pathway is inhibited, and cells are further cultured under supplements previously shown to support CLCs from hPSCs. Briefly, cardiac differentiation started at day (D) 0 (*D0*) by adding the GSK-3 β inhibitor CHIR in the presence of chemically defined media (RPMI/B27 -insulin). At this stage cell monolayers are expected to form homogenous nets. To promote optimal cardiac differentiation during early stages of cell culture, insulin was removed from the basal media until *D5*, as it has been described that the insulin/IGF pathway negatively regulates cardiac differentiation

(239). On the other hand, the spatio-temporal modulation of Wnt pathway, which is critical during heart development, is promoted from *D0* up to *D1* by the induction of mesendodermal/mesodermal specification making use of CHIR, a potential inducer of the Wnt pathway through the inhibition of GSK-3 β (240). Then, Wnt pathway is inhibited by the addition of IWP4 on *D3* of differentiation to promote cardiac lineage commitment. From *D5* insulin was supplemented in cardiac media and cells were subsequently maintained until *D30* in culture (Fig. 12A). During the onset of the experiments, CLCs started beating by *D12*. Taking advantage of *in vivo* and *in vitro* data from human heart development we selected 3 different cardiac target genes as surrogates of cardiac differentiation in our analysis (234) (Fig. 12B). The 3 cardiac target genes, *MYH6*, *MYL2* and *SIRPA* express at different time points during cardiac differentiation. According to these data, positive signals from GFP and mOrange fluorescent protein were assessed by live confocal imaging at the expected time-point, confirming our reporter cell lines as a precise tool for monitoring and tracing cardiac differentiation (Fig. 12C).

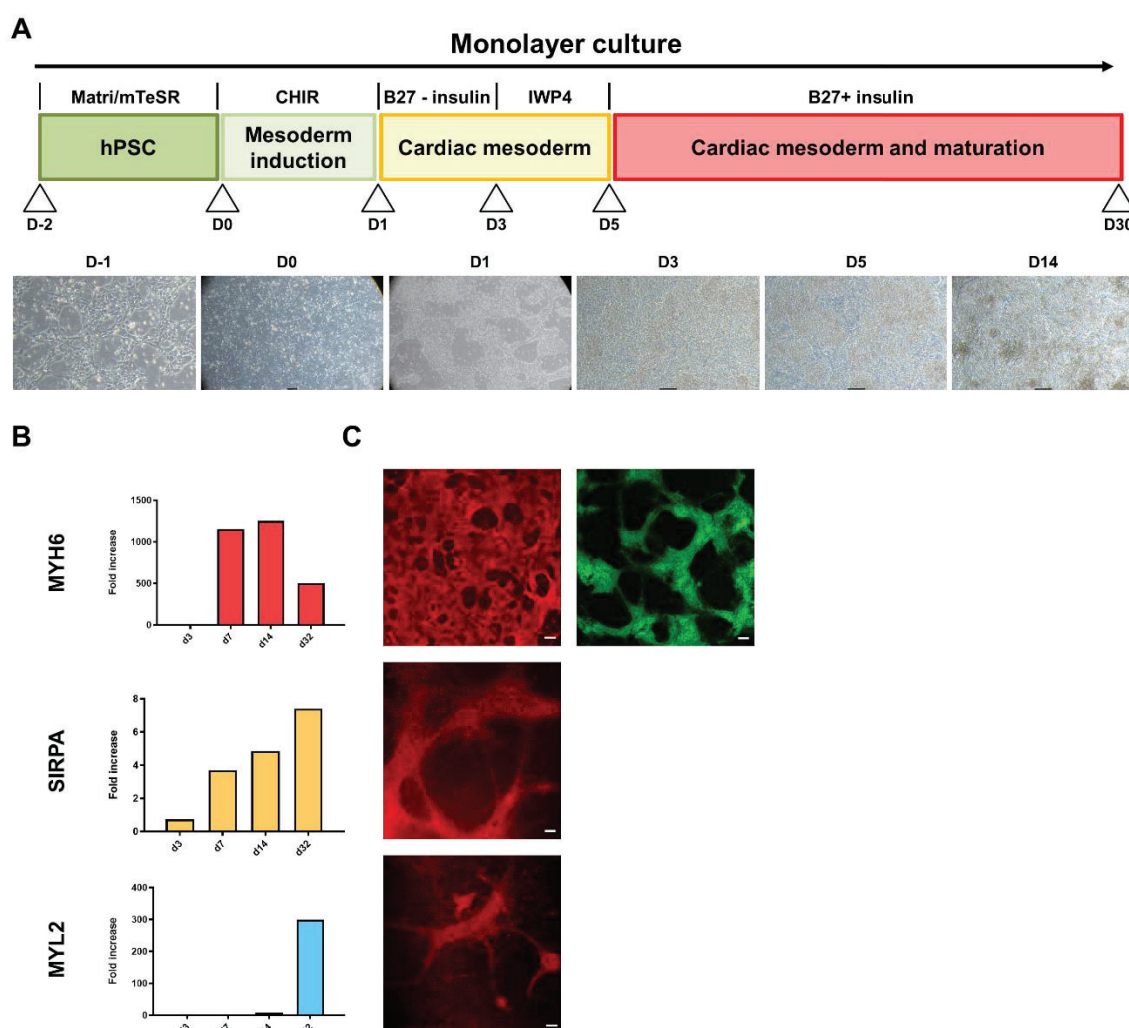


Fig. 12. Generation of CLCs from hPSC MYH6-GFP, hPSC MYH6-mOrange, hPSC MYL2-mOrange and hPSC SIRPA-mOrange in 2D. A) Experimental scheme for the differentiation of CLCs from hPSC: cardiac mesoderm was induced by activation and inhibition of Wnt pathway (CHIR and IWP4) and CLCs were maintained in culture taking advantage of B27/RPMI B27 + insulin media from D5 until D30 of differentiation. **B)** RNA bulk Seq data showing the expression of MYH6, MYL2 and SIRPA during our cardiac differentiation in D3,7,14 and 32. **C)** Fluorescent reporter expression during cardiac differentiation. Representative images of mOrange and GFP expression (MYH6 reporters) starting from D7 of cardiac differentiation, and mOrange specific for SIRPA (D21) and MYL2 (D30) through live imaging. Scale bar 100 μ m. CLCs were generated independently in 3 to 5 biological experiments.

4.2.2. Characterization of the cardiac differentiation potential of hPSCs cardiac reporter lines to support CLCs differentiation in 2D culture conditions by quantitative PCR analysis

To ensure that the process of genome editing did not compromise the overall differentiation potential of the generated cell lines, we subjected both parental hPSC and the resulting hPSC MYH6-GFP #C4 and hPSC MYH6-mOrange #G12 reporter

cell lines into our well-defined cardiac differentiation protocol. Samples were analyzed at specific time points during the onset of differentiation (*D0*, 3, 7, 14, 21 and 30) by qPCR for the mRNA expression of cardiac genes. Our results showed that all the analyzed genes exhibited similar patterns for expression. As expected, brachyury (*T*), marker for posterior streak fate, and *GATA4*, specific for the mesoendoderm, were highly expressed at *D3*. Conversely, cardiac genes *MYH6*, *MYL2* and *TNNT2* were expressed upon induction of the cardiac program (Fig.13). *MYH6* is the first cardiac marker that starts to express at *D7*. In contrast, myosin light chain 2 (*MYL2*), ventricular-specific marker of cardiac myocardium and troponin T (*TNNT2*), one of the components of the troponin complex playing a crucial role in regulating muscle contraction, are expressed during late-stages of differentiation. Overall, these data confirmed that *MYH6* targeting through CRISPR/Cas9 genome editing did not compromise the cardiac differentiation potential of the resulting reporter cell lines.

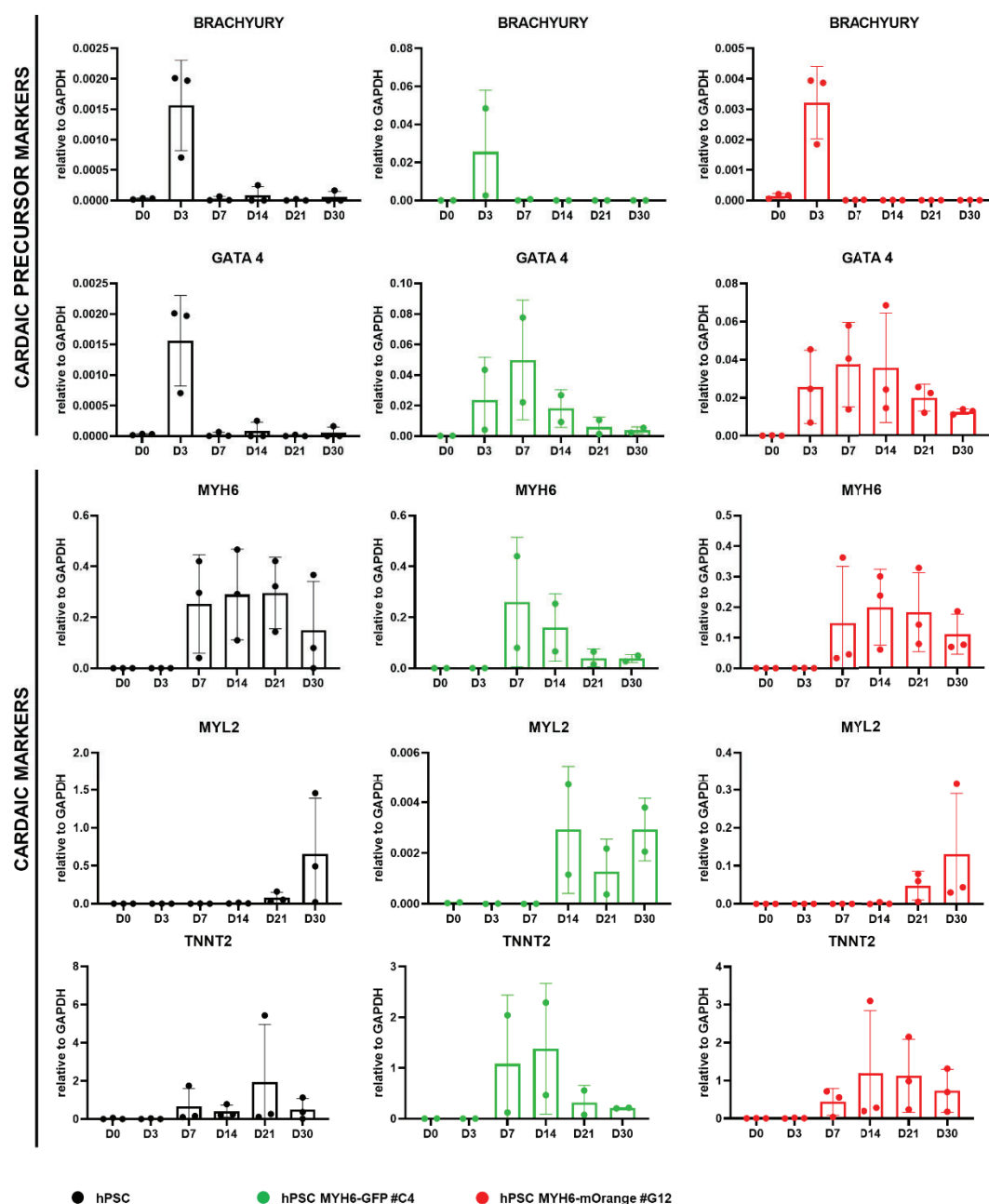


Fig. 13. Characterization of hPSC cardiac reporter lines differentiation in 2D by qPCR. Representative images of qPCR analysis for the indicated markers during the onset of cardiac differentiation (*D0* to *D30*). Analysis compares the expression of the same markers in hPSC (black), hPSC MYH6- GFP #C4 (green) and hPSC MYH6-mOrange #G12 (red) cell lines. *Data are mean $\pm$ SD. At least $n = 2$ independent biological experiments with mean of 3 technical replicates each.*

4.2.3. Characterization of the cardiac differentiation potential of hPSCs cardiac reporter lines to support CLCs differentiation in 2D culture conditions by confocal microscopy analysis

We next proceed to analyze a panel of cardiac-related markers by immunofluorescence in both hPSC MYH6-GFP #C4 and hPSC MYH6-mOrange #G12 reporter cell lines at late stages of differentiation. Our analysis revealed the expression of GFP and mOrange, both indicating MYH6 localization and Alpha Sarcomeric Actin (ASA). Troponin T (TNNT) and the cardiac fibroblast marker vimentin (VMT) were also localized in the same cells expressing the analyzed cardiac markers (Fig. 14). These cells were frequently found in areas where the tissue was beating, further supporting the functionality of the generated reporter cell lines and their proper differentiation towards CLCs. These results provide strong evidence of successful cardiac differentiation in all the reporter cell lines generated during this thesis.

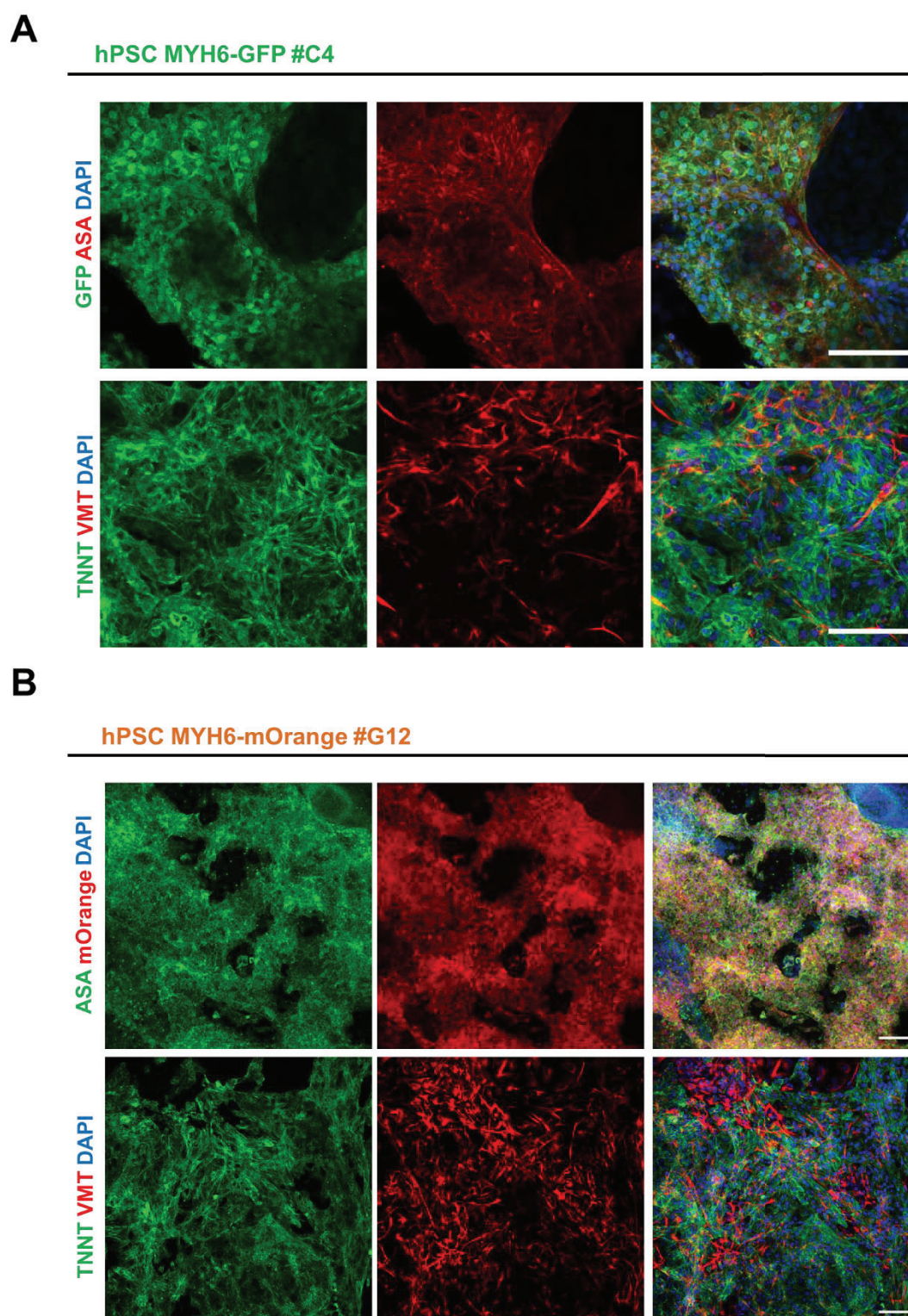


Fig. 14. Characterization of hPSC cardiac reporter lines differentiation in 2D by immunofluorescence analysis. **A)** Representative images of the immunofluorescence analysis performed in hPSC MYH6-GFP #C4 cell differentiated to CLCs to detect the expression of the cardiac markers GFP (green), Alpha Sarcomeric Actin (ASA, red), Troponin T (TNNT, green) and Vimentin (VMT, red) at late stages of cardiac differentiation. **B)** Representative images of the immunofluorescence analysis performed in hPSC MYH6-mOrange #G12 cell differentiated to CLCs to detect the expression of the cardiac markers Alpha Sarcomeric Actin (ASA, green), mOrange (red), Troponin T (TNNT, green) and Vimentin (VMT, red) at late stages of cardiac differentiation. Scale bar 100 μ m. At least $n=3$ independent biological experiments of *in vitro* characterization were performed.

4.3. Differentiation and characterization of hPSC cardiac reporter lines towards CLCs and resident cardiac-like cells in 3D cell culture conditions

Two-dimensional cell culture has been a fundamental tool in biological research for decades. It has contributed significantly to our understanding of cell biology, but its limitations are becoming increasingly apparent. One of the major limitations is its inability to represent advanced stages of differentiation, particularly in the context of complex tissues such as the native heart. To overcome this constraint, the development of 3D cell culture conditions is necessary, as they provide a more realistic and supportive environment for hPSCs, allowing them to recapitulate key aspects of tissue development, differentiation, and disease. This has led to the emergence of cardiac organoids, miniature models of the embryonic heart created through the exposure to 3D cell culture conditions. In this work, starting from the well-established 2D cardiac differentiation protocol already published in the host laboratory, we generated hPSC-derived self-organizing cardiac-like organoids to use as a model to better understand cardiac differentiation and set the bases for further studies modeling heart disease.

4.3.1. Establishment of cell culture conditions supporting the derivation of cardiac-like organoids

Based on our results on the potential use of hPSCs cardiac reporter lines to monitor cardiac differentiation in 2D cell culture conditions, we aimed to develop a high-throughput, reproducible, and relatively simple protocol for the generation of self-assembling cardiac-like cells in 3D culture conditions. To that we further leverage on hPSC MYH6-GFP #C4 reporter cell line. To set up the procedure we first relay on the derivation of mesoderm progenitors at *D3* as starting time point to force cell-to-cell and cell-to-ECM contact by the aggregation of cells making use of spin centrifugation. A comprehensive description of the protocol for the generation of cardiac-like organoids can be found in Materials and Methods (Section 3.3.2). Briefly, *D3* monolayers generated by the exposure of undifferentiated hPSC MYH6-GFP #C4 to 12 μ M CHIR were detached by enzymatic digestion into single cell suspensions that were spun down by centrifugation in 96-well plates. Subsequently, we further inhibit the Wnt pathway for 48 h using IWP4 factor. Afterwards, cardiac-like cells in 3D were cultured

in cardiac basal media (B27+insulin) up to *D90*. Notably, cardiac-like organoids exhibited spontaneous beating earlier than 2D CLCs, manifesting as early as *D8-10*. We assessed GFP expression as a surrogate of MYH6 expression by live confocal imaging, correlating it with endogenous *MYH6* expression at expected time points during differentiation (Fig. 15A). Bright field images at *D30* and *D60* showed the maintenance of a compact structure during the differentiation. H&E staining did not show significant differences between the two time points, differently from MT that at *D60* exhibited enhanced blue-coloured collagen deposition, suggesting the progressive development of extracellular matrix components necessary for tissue structure and support. During the differentiation process, there was a noticeable decrease in the size of the organoids. This observation was further supported by measuring the average size of around 200 cardiac-like organoids. To obtain the real size of the cardiac-like organoids an algorithm in MATLAB was used in a collaboration with Hospital General Universitario Gregorio Marañón in Madrid (231). This algorithm identified the contours of the organoids and successively counted the number of pixels within the mask, which was subsequently divided by the total number of pixels. (Fig. 15B).

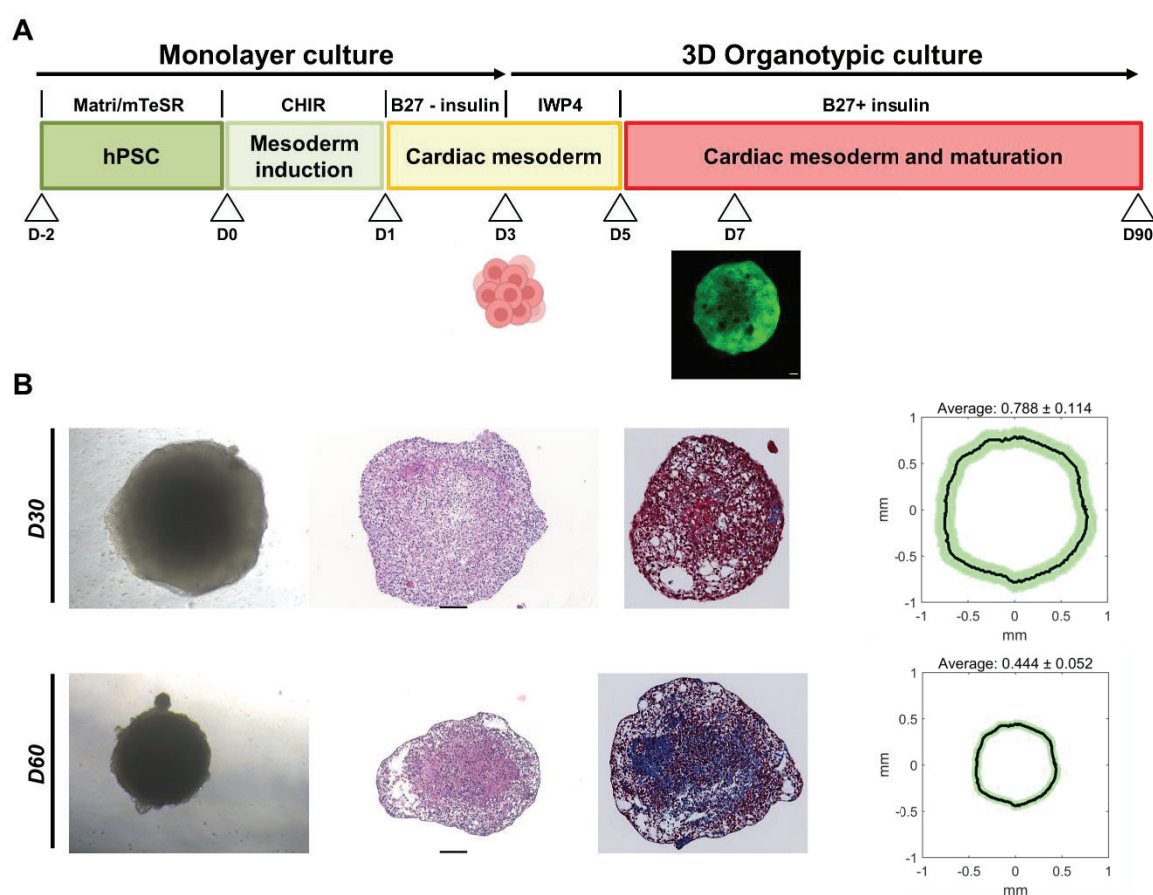


Fig. 15. Generation of cardiac-like organoids from hPSC. **A)** Experimental scheme for the differentiation of cardiac-like organoids from hPSC MYH6-GFP #C4: cardiac mesoderm was induced by activation and inhibition of Wnt pathway (CHIR and IWP4) such as in 2D conditions. Self-assembled 3D organoids were aggregated at *D3* and maintained in cell culture until *D90* of differentiation. Live image of GFP for the assessment of the fluorescent reporter expression in cardiac-like organoids at *D7*. Scale bar 100 μ m. **B)** Representative bright field, H&E and MT images of cardiac-like organoids at *D30* and *D60* with the respective average size. Average size of at least $n = 190$ cardiac-like organoids from 2 to 5 independent biological experiments.

4.3.2. Characterization of the cardiac reporter cell line hPSC MYH6-GFP #C4 differentiation in 3D culture conditions into cardiac-like organoids by quantitative PCR analysis

To thoroughly characterize our new procedure for generating self-assembling cardiac-like cells in 3D culture conditions, we performed qPCR analysis until *D90* to monitor expression markers related to mesoderm fate commitment, cardiac differentiation, ECM turnover, glycolysis, and mitochondrial respiration and function. Our results revealed the time-expected expression of key cardiac markers throughout the differentiation process, including *MYH6*, *MYL2*, *TNNT2*, and *TNNI3*. These markers are critical for cardiomyocyte differentiation and contraction. Interestingly, at *D90* there was a notable increase in the expression of *MYL2*, *TNNT2*, and *TNNI3*. Troponins (*TNNT2* and *TNNI3*) work together as part of the troponin complex to regulate the contraction and relaxation of cardiac muscle, whereas *MYL2* is the major sarcomeric protein in mammalian striated muscle fundamental in the regulation of myosin ATPase activity in smooth muscle, skeletal muscle, and cardiac muscle (241,242). This finding is particularly intriguing as it suggests a higher level of maturity in the cardiac-like organoids which is correlated with a more fully formed contractile apparatus. Meanwhile, *MYH6*, one of the essential proteins required for cardiomyocyte functionality is expressed homogeneously during all the differentiation. This finding is supported by the biological expression of this protein at both early and late stage of cardiac differentiation in the murine heart (89).

In addition to specific cardiac markers, we also analyzed the expression of different genes important for the electrical and mechanical properties of the cardiomyocytes. For instance, voltage-gated sodium channel Nav1.5 (*SCN5A*) mRNA shows a clear increase in expression in mature cardiomyocytes due to its role in allowing sodium influx and membrane depolarization (243). Conversely, Cav1.2 core component

(*CACNA1C*) is highly expressed in immature cardiomyocytes compared to mature counterparts, due to its role reducing the *plateau* phase of action potential (87).

Furthermore, it is worth noting that the process of cardiomyocyte differentiation is accompanied by a significant shift in metabolic activity, as shown in pioneering studies by Yanes et al. (244). Metabolic transcriptional regulators such as Peroxisome Proliferator Activated Receptor Alpha (*PPARα*) and lactate dehydrogenase A (*LDHA*) are activated during the process of differentiation, driving to a higher expression of genes involved in fatty acid metabolism, oxidative phosphorylation, and mitochondrial biogenesis (104,105). Hexokinase isoform 2 (*HK2*) is a glycolytic marker more abundant in late stage showing low glycolytic activity (107). On the other hand, uncoupling protein 2 (*UCP2*) tends to decrease at late stages. It is a protein belonging to the family of mitochondrial anion carrier plays an important role against cellular dysfunction by reducing mitochondrial oxidative stress (245). Finally, we also analyzed the endothelial markers, *CD31* and *ENDOGLIN*, showing appearance of vascularization around *D30* in cardiac-like organoids.

Taken together, qPCR analysis provided comprehensive characterization of our new procedure supporting the derivation of cardiac-like organoids from hPSCs. Overall, our results confirm the expression of specific markers crucial for cardiac and endothelial cell fates, as well as metabolic performance. Our findings not only demonstrate the effectiveness of our approach, but also provide insights into the molecular mechanisms underlying the differentiation of self-assembling cardiac-like cells in 3D culture conditions (Fig. 16).

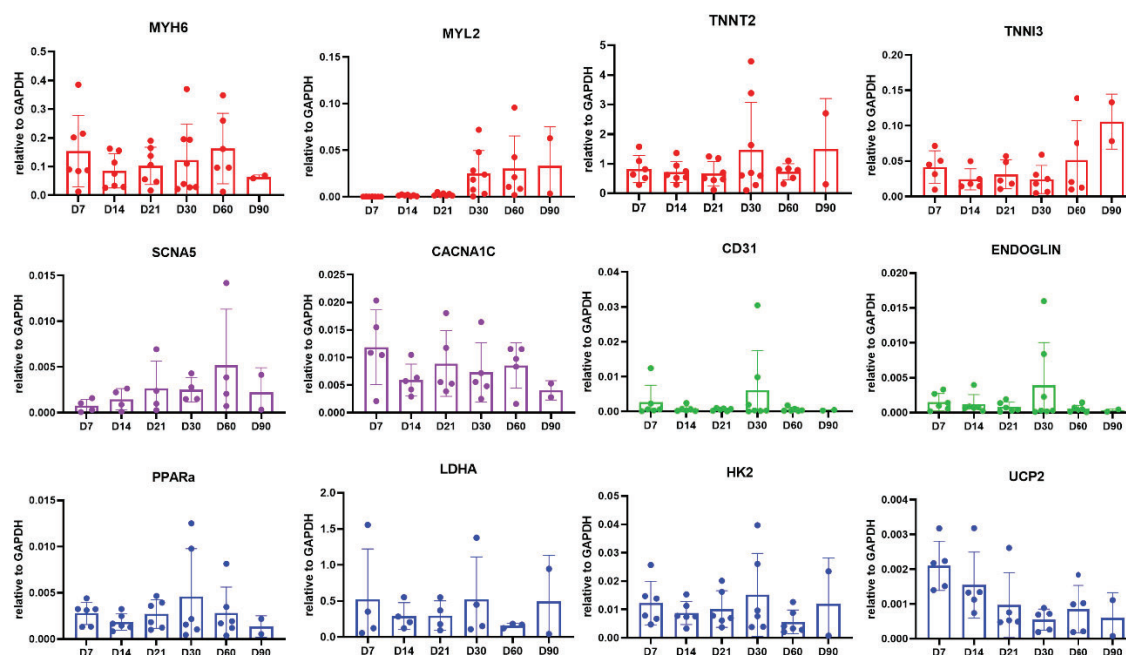


Fig. 16. Characterization of the cardiac reporter cell line hPSC MYH6-GFP #C4 differentiation in 3D culture conditions into cardiac-like organoids by quantitative PCR analysis. Representative images of qPCR analysis for the indicated markers during the onset of differentiation from the generation of cardiac-like organoids (*D7* to *D90*). The expression of specific cardiac markers corresponds to the expected time frame for their expression (red). Additionally, the analysis of cardiac ion channel (purple), endothelial (green) and metabolic (blue) markers. Data are mean $\pm$ SD. $n = 5$ to 7 independent biological experiments from a pool of 12 organoids/group with mean of 3 technical replicates each.

4.3.3. Characterization of the cardiac reporter cell line hPSC MYH6-GFP #C4 differentiation in 3D culture conditions into cardiac-like organoids through confocal microscopy analysis

To further assess the efficacy and robustness of the approach described in this thesis, we performed immunofluorescence analysis at different time points during cardiac-like organoids differentiation. Like the 2D condition, we detected GFP expression together with the endogenous expression of MYH6 from *D7* until the end of the differentiation process (*D90*). Moreover, as shown in Fig. 16, qPCR analysis highlighted the detection of *CD31* mRNA from *D30*. This finding correlates with the detection of CD31 by confocal microscopy analysis suggesting the spontaneous formation of vascular-like structures within hPSCs-derived cardiac-like organoids (Fig. 17A). Taking advantage of our experimental design, we explored the expression of different markers during different time points of differentiation, specifically at *D30*, *60* and *90*. Notably, we observed the maintenance of CD31 detection until *D90* by immunofluorescence

analysis. Moreover, our results highlighted the detection of troponin T (TNNT), a key component of the thin filament regulatory complex that imparts calcium-sensitivity to striated muscle actomyosin ATPase activity (Fig. 17B). Furthermore, the detection of connexin 43 (CX43) indicated the presence of gap junction channels well-known for the propagation of electrical depolarization in native cardiomyocytes (Fig. 17C). Interestingly, we conducted a more detailed analysis for the detection of α -Sarcomeric Actin (ASA), a marker specific for α -skeletal muscle actinin and α -cardiac actinin. It stains z-disc and dots in stress fibers of myotubes in skeletal and cardiac muscle but not in non-sarcomeric muscle element(s) (Fig. 17C).

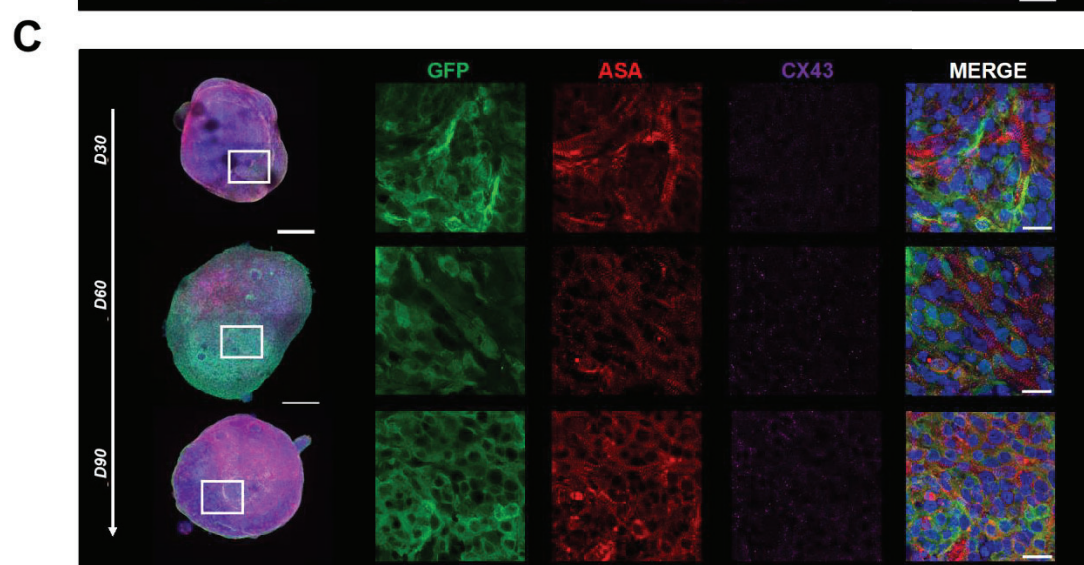
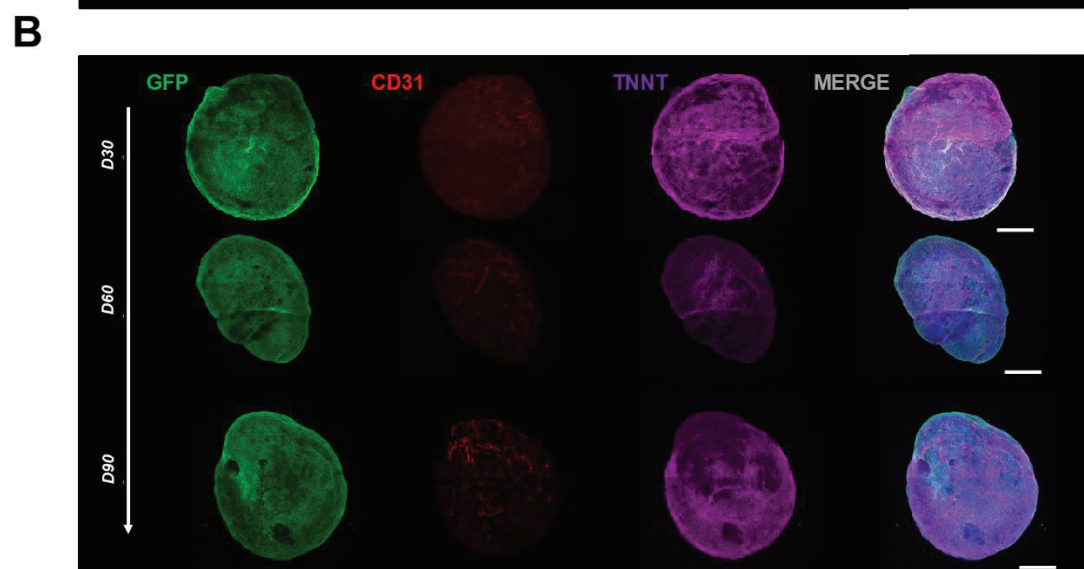
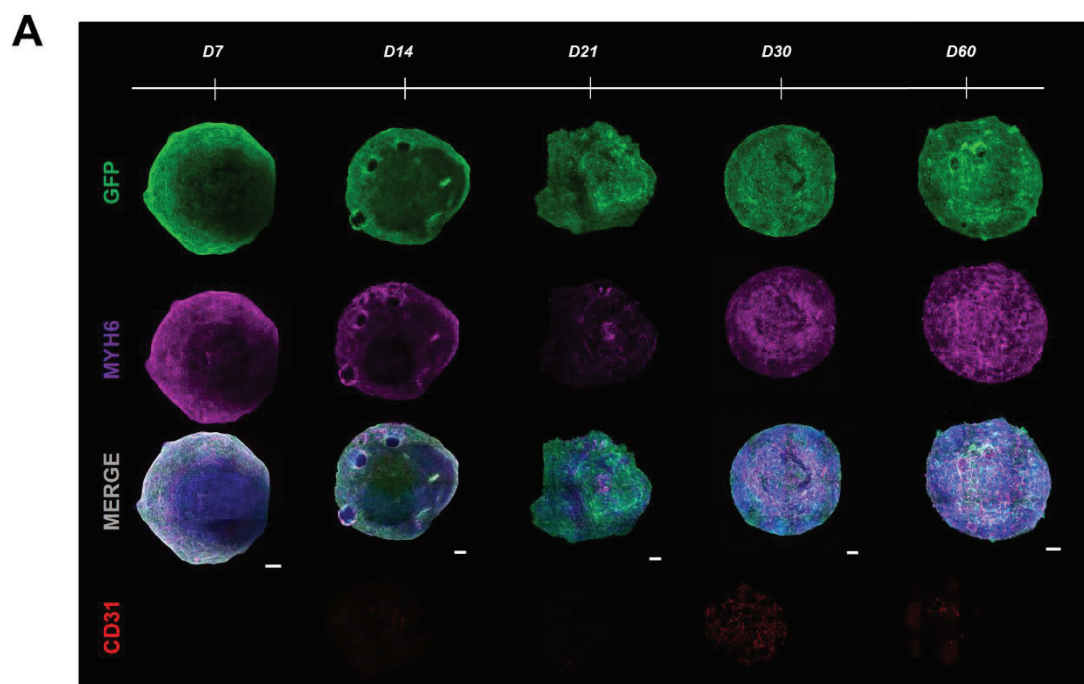


Fig. 17. Characterization of the cardiac reporter cell line hPSC MYH6-GFP #C4 differentiation in 3D culture conditions into cardiac-like organoids through confocal microscopy analysis. A) Representative images of the immunofluorescence analysis performed in cardiac-like organoids in the hPSC MYH6-GFP #C4 cell line to assess the expression of MYH6 (far-red) and GFP (green) from *D7* until *D60* of cardiac differentiation. Detection of endothelial marker CD31 (red) from *D30*. *Scale bar 250 μ m.* **B)** Representative images of the immunofluorescence analysis performed at *D30,60* and *90* of cardiac-like organoid differentiation highlight the detection of GFP (as a surrogate of MYH6 expression, green), CD31 (red), TNNT (far-red). *Scale bar 250 μ m.* **C)** Representative images of details for the detection of GFP (green), ASA (red) and CX43 (far-red). *Scale bar 250 μ m in full organoids and 20 μ m in magnified images.* Representative images from a total of at least $n=2$ organoids from at least $n=3$ independent biological experiments. Similar results were obtained in 2 to 5 independent biological experiments.

It is widely recognized that sarcomeres are generally longer in mature cardiomyocytes compared to their immature counterparts (246). The increase in sarcomeric length is associated with an increased contractile force that enables the heart to pump blood more efficiently during heart development and after injury in several animal models (247). In this regard, we measured the sarcomeric length based on immunofluorescence staining for ASA (Fig. 18A). Specifically, intensity profiles from adjacent α -actinin labelled filaments (z-disc) allowed the calculation of sarcomeric length as the distance between fluorescent intensity peaks (Fig. 18B). Z-disc thickness was automatically analyzed, and the mean width of each filament was calculated giving us the value of sarcomeric length. The range of sarcomeric length in our cardiac-like organoids ranged from 1.3 μ m and 1.8 μ m (Fig. 18C). These results align with published findings that have confirmed the limited range of sarcomeric lengths, typically falling between 1.2 μ m and 2 μ m (127,248). Moreover, it is notable that there is an increase in length from *D30* to *D60*. In contrast, *D90* showed a wide range of length. Importantly, it should be noted that *D30* and *D60* cardiac-like organoids derived from the same biological experiment, whereas *D90* cardiac-like organoids originated from a different biological experiment. These data highlights that our approach sustains for the formation of cardiac-like organoids that undergo structural maturation when cultured for long time.

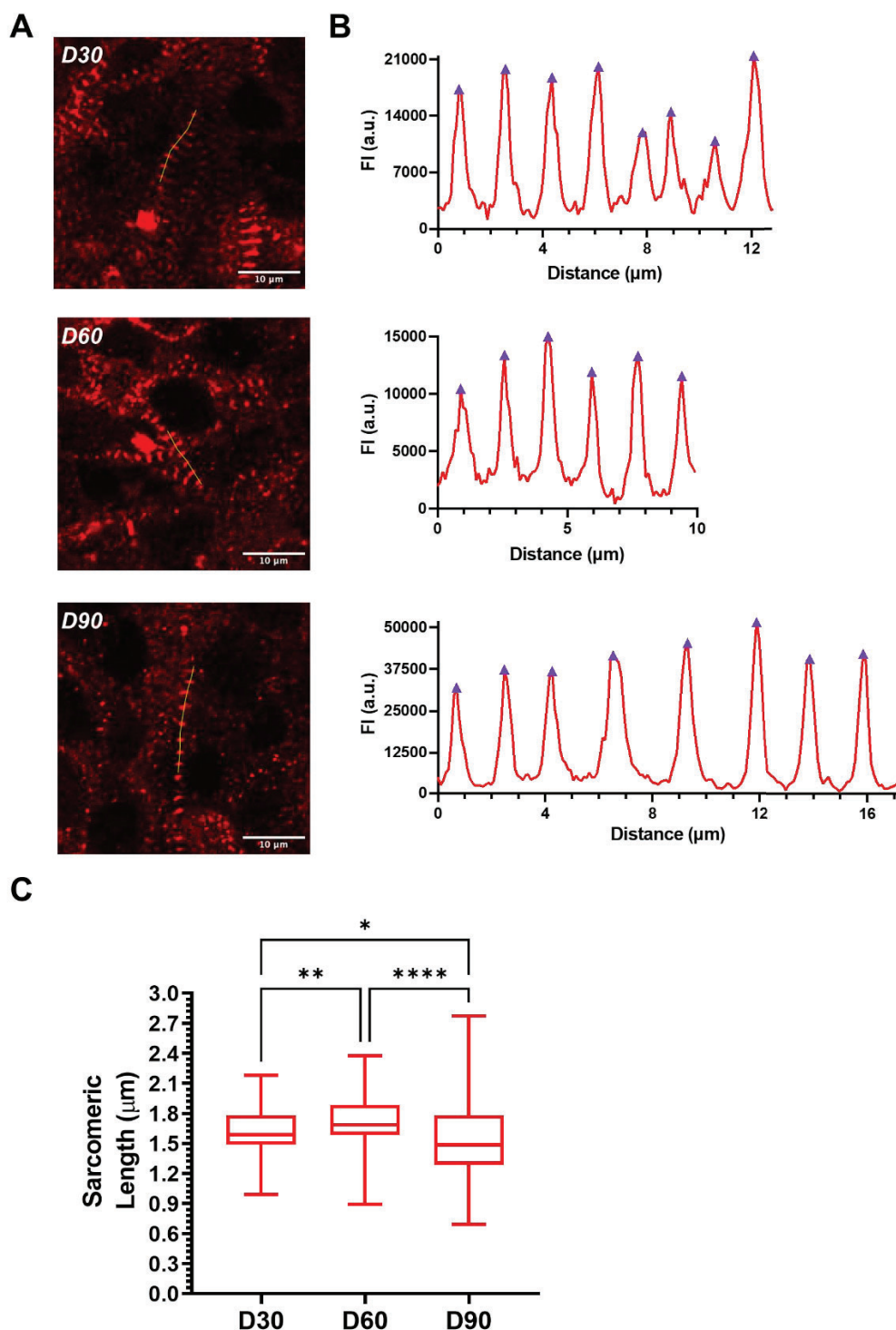


Fig. 18. Analysis of sarcomeric length in D30,60 and 90 cardiac-like organoids. **A)** Representative images of details for the detection of ASA (red) and in yellow, z-lines in a sarcomere manually traced with ImageJ. Scale bar 10 μm . **B)** Representative image of intensity profile graphs showing intensity peak corresponding to each z-disc. **C)** Sarcomeric length quantification by measuring the distance between intensity peaks derived from adjacent α -actinin labelled filaments. D30 and D60 cardiac-like organoids derive from the same biological experiment, D90 derives from different biological experiment. At least $n=34$ cells were analysed at each time condition. For each cell analyzed, at least 4 sarcomeres were measured, leading to at least 157 sarcomeres analyzed across all cells from 2 to 5 independent biological experiments. Statistical analysis used for this analysis were One-way Anova and multiple comparison Tukey's tests.

4.3.4. Characterization of the cardiac reporter cell line hPSC MYH6-GFP #C4 differentiation in 3D culture conditions into cardiac-like organoids through transmission electron microscopy

Ultrastructural analysis of cardiac-like organoids at *D30* and *D60* showed similar features to those found in the human fetal heart, with well-defined sarcomeres surrounded by mitochondria, gap junctions and the presence of tubular structures reminiscent of T-tubules (Fig. 19A). Comparing early (*D30*) and late (*D60*) stages of differentiation, transmission electronic microscopy analysis (TEM) also highlights the presence of mitochondria and improved alignment of sarcomere filaments in *D60* samples compared to *D30*. The presence of T-tubules was also assessed at *D30* and *D60* by immunofluorescence analysis using wheat germ agglutinin (WGA). WGA label and binds glycoproteins of the cell membrane, commonly used for the staining of skeletal and cardiac sarcolemma. Indeed, T-tubules are invaginations of the cardiomyocyte sarcolemma playing a crucial role in various physiological processes (i.e. excitation- contraction coupling, maintenance of resting membrane potential, intracellular signaling transduction) (249). In our cardiac-like organoids, T-Tubules were observed surrounding the nucleus (DAPI) and sarcomeres (ASA), showing a more compact structure at *D30* (Fig. 19B).

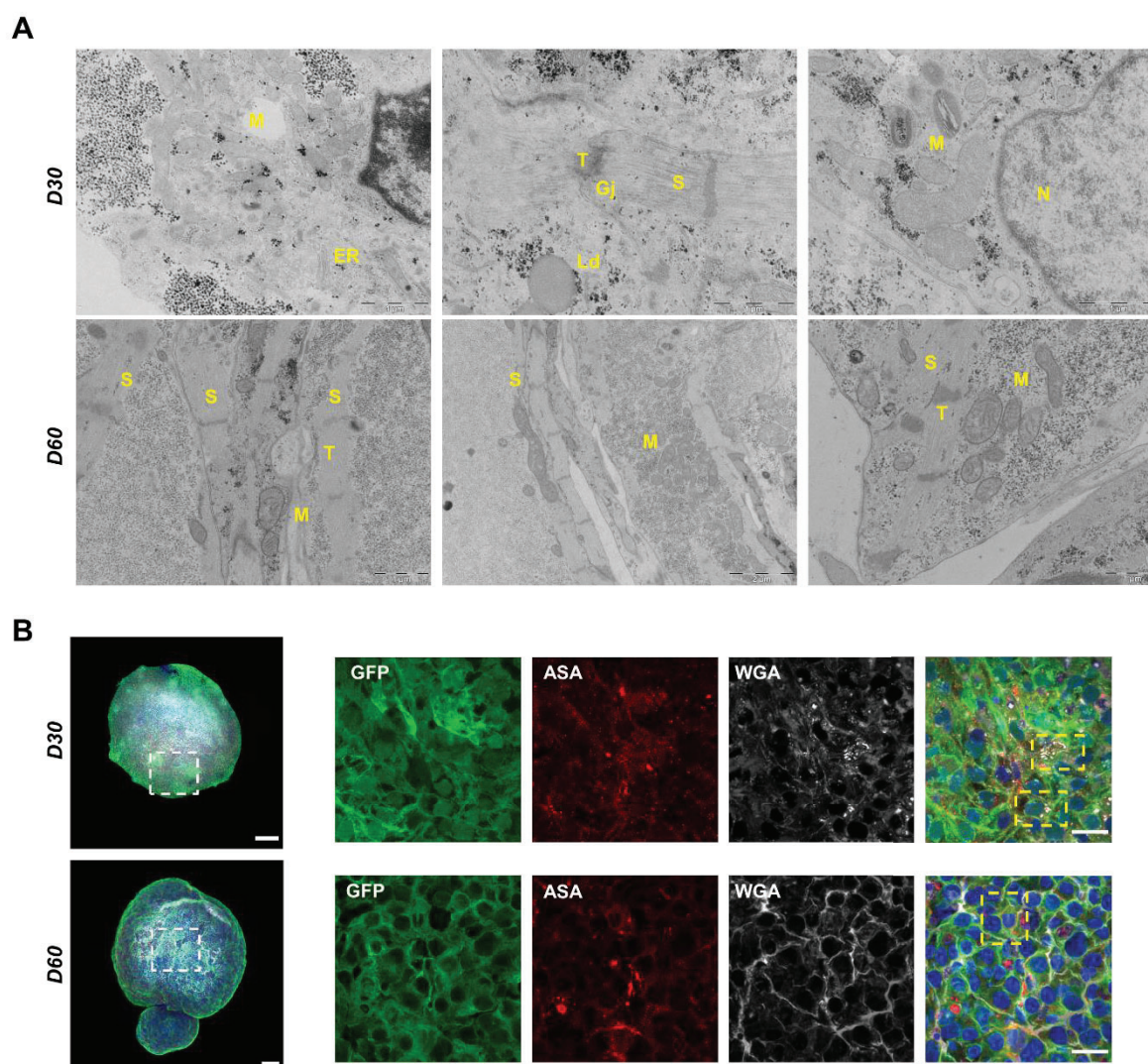


Fig. 19. Characterization of the cardiac reporter cell line hPSC MYH6-GFP #C4 differentiation in cardiac-like organoids by TEM. **A)** Representative TEM images of cardiac-like organoids at D30 and D60 showing endoplasmic reticulum (ER), gap junction (Gj), mitochondria (M), nucleus (N), lipid droplets (Ld), T-tubules (T) and sarcomeres (S). Scale bar 1 μm and 2 μm . **B)** Representative images of the immunofluorescence analysis performed at D30 and 60 of cardiac-like organoid differentiation highlight the detection of GFP (as a surrogate of MYH6 expression, green), ASA (red) and WGA (grey). Scale bar 250 μm in full organoids and 20 μm in magnified images. Representative images from a total of at least $n=2$ organoids from at least $n=3$ independent biological experiments.

4.3.5. Characterization of the cardiac reporter cell lines hPSC MYH6-mOrange #G12 and hPSC MYH6-GFP #C4 differentiation in 3D culture conditions into cardiac-like organoids for the assessment of functional activity

Electrophysiological analysis at different time points of cardiac differentiation in 3D culture conditions were performed in both hPSC MYH6-mOrange #G12 and hPSC MYH6-GFP #C4 in a collaboration with Hospital General Universitario Gregorio Marañón in Madrid by Antonio Sanchez Salgado under the supervision of Gonzalo

Rios Muñoz (231). Representative images of calcium transient mapping displayed the variation over time reflecting the entry and exit of calcium from the cells in hPSC MYH6-mOrange #G12 at *D18*, *D31* and *D60* and in hPSC MYH6-GFP #C4 cardiac-like organoids at *D21*, *D30*, *D60* and *D62*. These results are represented with the respective signal analyzed by OM Analyzer (MATLAB App) recording the voltage of each cardiac-like organoid during the time (Fig. 20A and 20C). Moreover, calcium transient duration measurements are displayed, the durations of calcium transient from t0 to 50% (CaT50) and 70% (CaT70) of repolarization (calcium extrusion) are calculated in both cell lines stimulating the cells at 1Hz, 2Hz and 3Hz. Subsequently, a final basal acquisition was recorded. Once all cells were analyzed, flecainide was administered. Flecainide is a Nav1.5 inhibitor, acting as antiarrhythmic drug. As expected, the administration of flecainide increased the duration of the overall calcium transient signal, promoting its antiarrhythmic effect in the samples. Overall, these data showed that in hPSC MYH6-mOrange #G12 cell line the effect was greater in *D18* and *D31* for both CaT50 and CaT70. The effect on *D60* cardiac organoids also prolonged the calcium transient duration. The electrophysiological characterization protocol conducted at different stimulation frequencies of 1-3Hz reported shorter duration of CaT50 and CaT70 for *D18* and *D60*. Stimulated recordings for the *D31* reported low to no variation at all in the CaT50 or CaT70 durations (Fig. 20B). In the case of hPSC MYH6-GFP #C4 cell line, the CaT50 and CaT70 durations were obtained at *D21*, *30*, *60* and *62*. The flecainide effect prolonged the calcium transient duration in all the groups, except for the youngest *D21*. For the rest of the groups, *D30*, *60* and *62*, there was a visible antiarrhythmic effect in the CaT50 and CaT70 duration. The stimulation protocol was also found less effective in *D21* than in *D30*, *60* and *62* (Fig. 20D). Both *D30* and *D60* showed higher frequency coupling, with *D60* showcasing more precise and consistent responses to stimulation frequencies. *D60* exhibited abbreviated CaTD values in comparison to the other groups, signifying expedited calcium transient dynamics and more efficient contraction-relaxation processes, suggesting a more mature stage of differentiation.

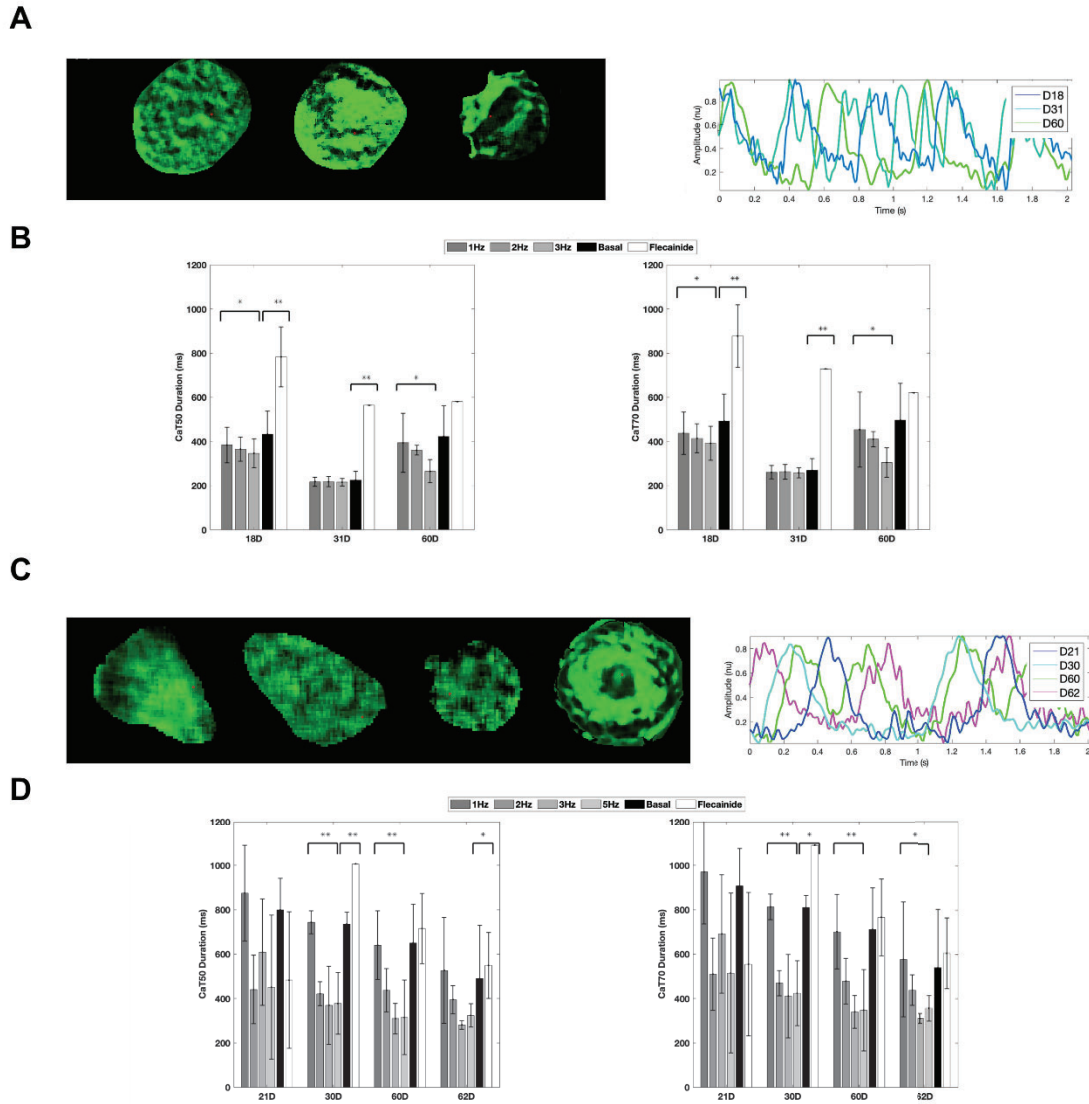


Fig. 20. Simultaneous optical mapping of transmembrane voltage and intracellular transient calcium of hPSC MYH6-mOrange #G12 and hPSC MYH6-GFP #C4 cardiac-like organoids. A) Representative images of calcium transient flux in hPSC MYH6-mOrange #G12 cardiac-like organoids at *D18*, *D31* and *D62* with the respective contraction recording. **B)** Optical mapping calcium transient CaT50 (left) and CaT70 (right) durations for the hPSC MYH6-mOrange #G12 at *D18*, *31* and *60*. Optical mapping images were acquired at basal condition, at different stimulation frequencies, 1-2-3-5 Hz, and after flecainide administration. Stimulated groups were analyzed with a bivariate statistical test, and basal and flecainide data with a two-tailed t-students test. (*) p-value <0.05; (**) p-value<0.001. *Data are mean of 24 to 36 organoids from n= 1 independent experiment.* **C)** Representative green fluorescent images of calcium transient flux in hPSC MYH6-GFP #C4 cardiac-like organoids at *D21*, *30*, *60* and *62* with the respective contraction recording. **D)** Optical mapping calcium transient CaT50 (left) and CaT70 (right) durations for the hPSC MYH6-GFP #C4 at *D21*, *30*, *60* and *62*. Optical mapping images were acquired at basal condition, at different stimulation frequencies, 1-2-3-5 Hz, and after flecainide administration. Stimulated groups were analyzed with a bivariate statistical test, and basal and flecainide data with a two-tailed t-students test. (*) p-value <0.05; (**) p-value<0.001. *Data are mean of 24 to 36 organoids from n= 1 independent experiment.*

4.3.6. Characterization of the cardiac reporter cell line hPSC MYH6-GFP #C4 differentiation in 3D culture conditions into cardiac-like organoids by single cell RNA Sequencing at *D30* and *D100*.

To deeper investigate the composition of our cardiac-like organoids and their level of maturation, in collaboration with Centro de Investigación Médica Aplicada (CIMA) in Pamplona, a whole-transcriptome analysis by scRNA Seq was performed at *D30* and *D100*. The uniform manifold approximation and projection (UMAP) analysis allowed the clustering of 11 populations confirmed by known cell type-specific markers. *D30* is characterized above all of cardiomyocytes, whereas at *D100* there is a clear maturation toward more specific cell types, such as atrial and ventricular, together with a small cluster of fibroblast and endothelial cells (Fig 21A). Moreover, a further investigation of the heterogeneity of our cardiac-like organoids was performed by analyzing the expression levels of specific genes for ventricular (MYL3, MYH7, HEY2, IRX1), atrial (NPPA, SHOX2), proliferative (PCLAF), endothelial (ESM1) and fibroblast (COL1A1) markers by violin plot and feature UMAPs. In these graphs we can see the switch from one of the myosin light chain class proteins (MYL3) at *D30*, marker for cardiomyocyte maturation, to a more cardiac specific genes, such as natriuretic peptide precursor-A (NPPA) and cardiac troponin I (TNNI3), specific for atria and cardiac muscle specification, respectively. Interestingly, high expression of COLA1A1 marks the small population of cardiac fibroblast cells at *D100* (Fig. 21B). The top 10 genes of each cell-type were analyzed by a heat map. At *D30*, the heat map demonstrates a higher expression of genes associated with cardiomyocytes, including interferon and proliferating cardiomyocytes. This indicates that at this stage of development, there is an active proliferation of cardiomyocytes and an elevated presence of interferon-related genes, possibly indicating immune response or cellular stress. Indeed, at *D100*, the heat map displays a contrasting pattern with higher expression levels observed in genes related to ventricular, atrial, interventricular, sinoatrial and fibroblast cell types (Fig 22). This suggests that the cardiomyocytes have undergone maturation and differentiation, leading to the emergence of more specific cell types within the cardiac-like organoid.

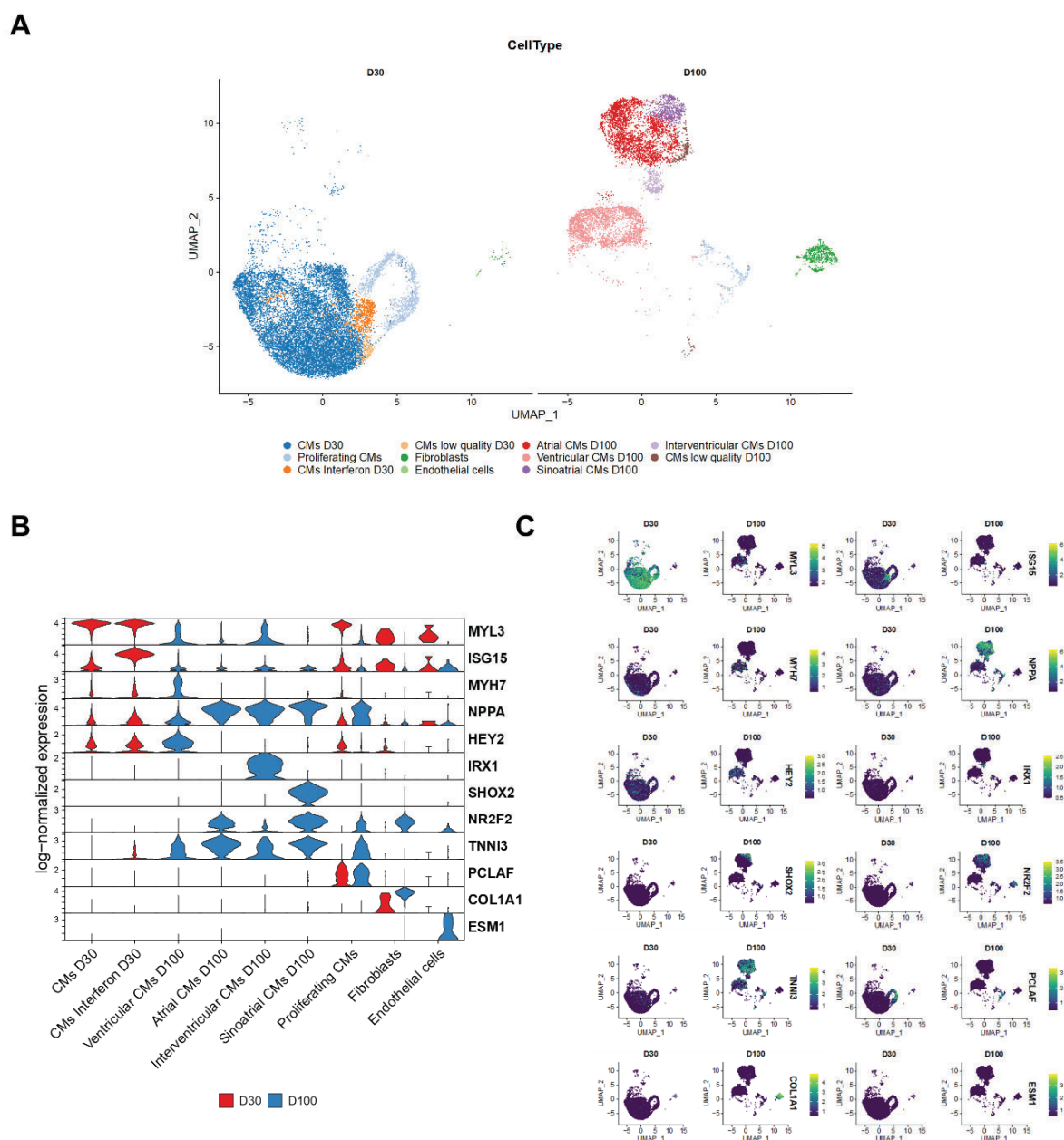


Fig. 21. scRNA Seq analysis of hPSC MYH6-GFP #C4 cardiac-like organoids at D30 and D100. A) UMAP plot showing cell clusters obtained by scRNA Seq of cardiac-like organoids at D30 and D100. Cells are color-coded according to their cell-type annotation using unsupervised clustering after integration, separating for the annotation non-proliferating CMs by day of differentiation. **B)** Violin plots showing the expression levels of genes which are differentially expressed in the cell-types split day of differentiation. **C)** UMAPs representing the log-normalized expression of cell-type markers split by sample of origin. Cells are color-coded based on expression level. Data are mean of $n = 48$ organoids.

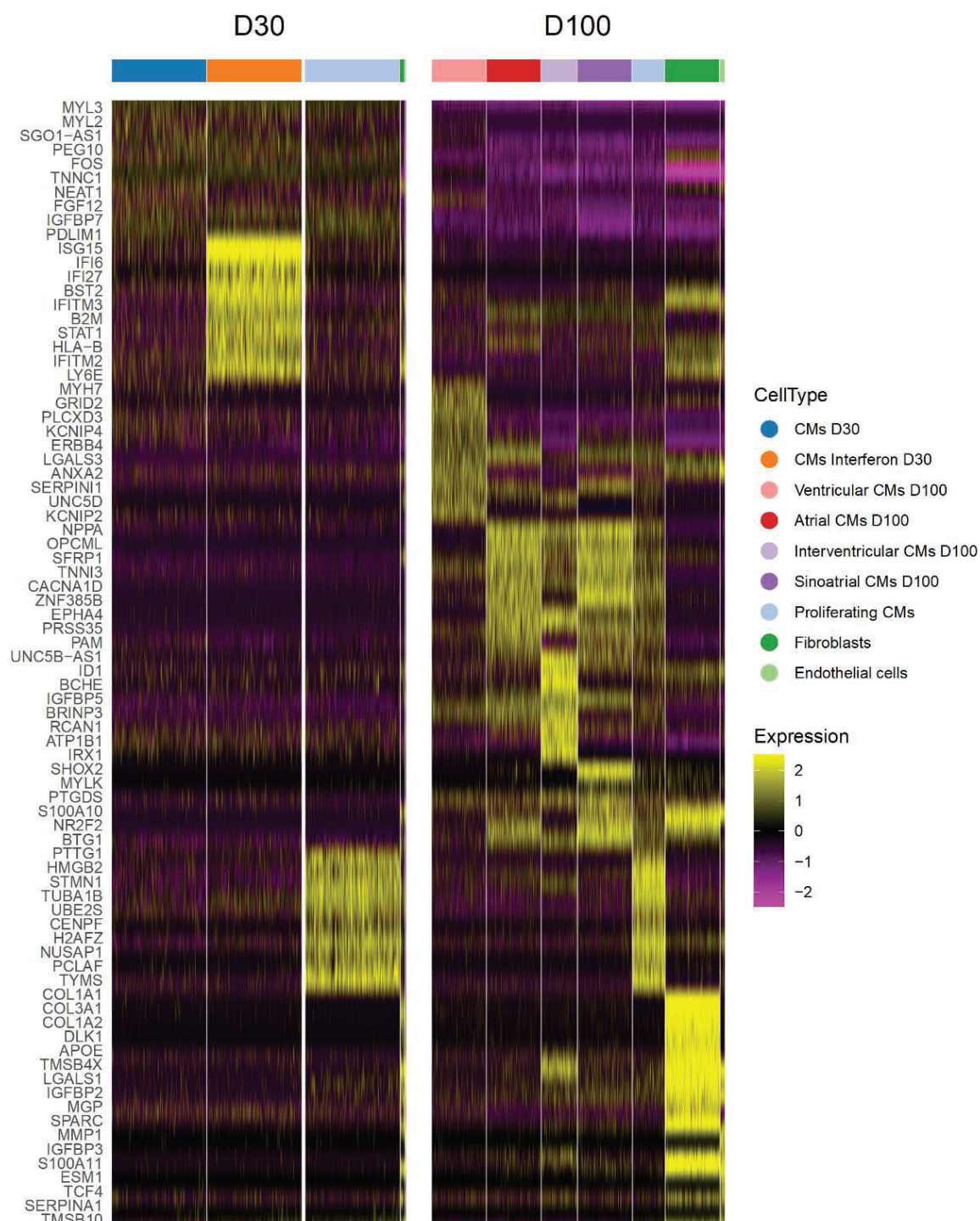


Fig. 22. scRNA Seq analysis of hPSC MYH6-GFP #C4 cardiac-like organoids at D30 and D100. Heatmap representing single cell scaled log-normalized expression for the top 10 markers of each cell-type split by day of differentiation. Only a maximum of 500 cells are represented per cell-type to better visualize expression in less abundant cell-types. Data are mean of $n = 48$ organoids.

Chapter II: Establishment of an *in vitro* platform based on the generation of cardiac-like organoids to study early hallmarks of diabetic disease

4.4. Establishment of cell culture conditions mirroring a normoglycemic and oscillatory glucose microenvironments in cardiac-like organoids derived from hPSCs cardiac reporter line

Diabetes is a prevalent multi-systemic disease characterized by elevated glucose levels, which may result from inadequate insulin production or the body's resistance to insulin. As already mentioned, diabetes can affect several organs bringing to secondary diseases, such as diabetic nephropathy (DN) and diabetic cardiomyopathy (DC). Animal models, in particular mice models, have been fundamental for the study and first treatments of diseases like diabetes (250). Mice models allowed the discovery of the available drugs treating the symptoms, but there is still no cure. For this reason, the need of new disease models allowing a better understanding of the pathology of diabetes. Organoids are the emerging experimental model that leverages many benefits of animal models. To date, several organoid models have been exploited to recapitulate disease relevant phenotypes, including genetic disorders and systemic conditions as diabetes (251). In this regard, the host laboratory pioneered the use of kidney organoids from hPSCs to model early hallmarks of diabetic disease (227). Here, to extend the use of our new procedure sustaining for the generation of cardiac-like organoids from hPSCs we have further investigated on the applicability of this *in vitro* platform not only to model cardiac development, but also to study early hallmarks of diabetic disease present in cardiac tissue. The host laboratory has previously found that applying a consecutive (7 days) exposure of kidney organoids into oscillating glucose concentration (from 5 to 25 mM) leads to an anomalous deposition of ECM (namely higher detection of collagen deposition in diabetic-like hPSCs-kidney organoids compared to control-normoglycemic counterparts) and to an overall metabolic rewiring similar to that found in kidney cells from Type 2 Diabetes patients (227). Here we took advantage of a similar approach to establish a new *in vitro* platform to model diabetic disease in cardiac-like organoids. All the experiments in this chapter were performed in the hPSC MYH6-GFP #C4 cell line. First, we dissect the profile of mitochondrial respiration of hPSCs-derived cardiac-like organoids to assess

proper time points to perform cell culture interventions promoting a diabetic-like response. To achieve this aim, we first performed Seahorse analysis as a feasible read out of mitochondrial fitness during cardiac-like organoid differentiation. We observed higher oxygen consumption rates at *D21* compared to *D12-30-70* together with higher basal respiration, ATP production and a decreased maximal respiration (Fig. 23A). Based on these observations we further selected *D21* as starting point to interrogate for the impact of normoglycemic (5 mM glucose), diabetic-like (5 to 25 mM glucose) as well as differentiation culture conditions supporting cardiac-like organoids differentiation (11 mM glucose) (Fig. 23B). First, experiments were conducted to analyse the impact of the different culture conditions in the overall extent of cardiac differentiation. To that we conducted qPCR and immunofluorescence analysis in cardiac-like organoids exposed to all the above-mentioned cell culture conditions. mRNA expression levels for specific cardiac markers during the onset of differentiation, such as myosins (*MYH6* and *MYL2*) and troponins (*TNNT2* and *TNNI3*), showed a similar trend of expression in both normoglycemic and diabetic-like conditions compared with the differentiation culture condition after one week of treatment (Fig. 23C). Our hypothesis receives further confirmation as we observe the consistent presence of GFP and ASA in all the conditions through immunofluorescence and confocal microscopy analysis (Fig. 23D). All in all, our results indicate that the induced diabetogenic environment did not directly affect the cardiac differentiation process.

Interestingly, the expression of the endothelial marker CD31 appears to be notably higher in the diabetic-like condition through confocal analysis compared to normoglycemic condition. It is widely established that both diabetes and insulin resistance can cause endothelial dysfunction. This dysfunction can result with upregulation expression of specific markers from endothelial cells, such as *CD31* and *ENDOGLIN*, data supported also by qPCR analysis' results (252) (Fig. 23E).

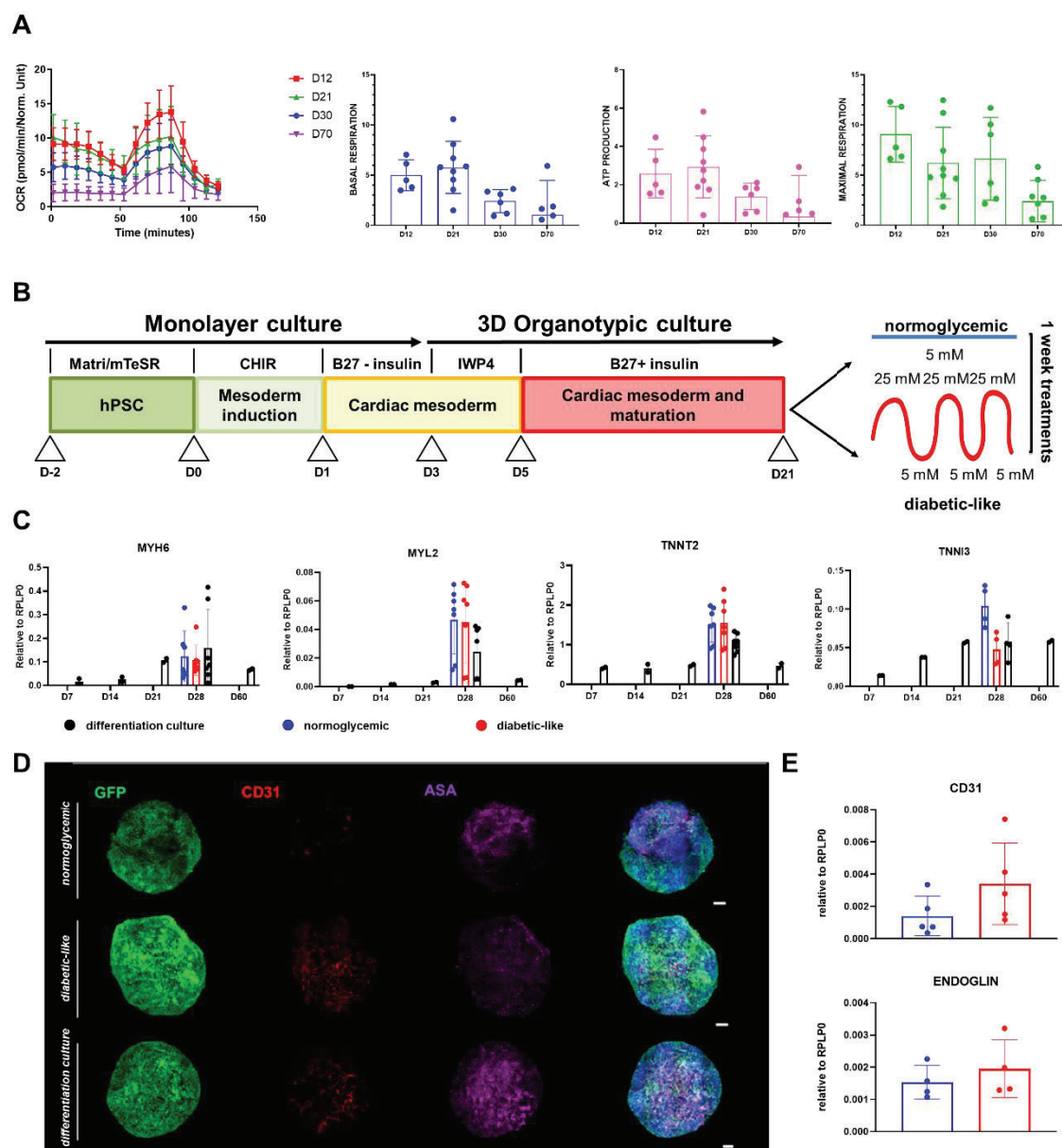


Fig. 23. Establishment of cell culture conditions mirroring a normoglycemic, diabetic-like and differentiation culture conditions in cardiac-like organoids. A) Sea horse analysis at different time points during cardiac-like organoid differentiation. The oxygen consumption rate (OCR) data are normalized to total protein. Basal respiration (blue), cellular ATP production (pink), and maximal respiration (green) are shown. *Data are mean $\pm$ SD. At least $n = 5$ independent replicates/group from $n = 2$ independent experiments.* **B)** Experimental scheme for the generation of human cardiac-like organoids from hPSCs with the oscillatory glucose stimulus from D21. **C)** Representative images of qPCR analysis for the indicated cardiac markers at D28, after one week of oscillatory glucose microenvironment, in cardiac-like organoids. Analysis compares the expression of the same markers in normoglycemic (5 mM glucose, blue), diabetic-like (5 to 25 mM glucose, red) and differentiation culture conditions supporting cardiac-like organoids differentiation (11 mM glucose, black). *Data are mean $\pm$ SD. At least $n = 5$ independent biological experiments from a pool of 12 organoids/group with mean of 3 technical replicates each.* **D)** Representative images of the immunofluorescence analysis performed in normoglycemic and diabetic-like conditions at D28 to confirmed expression of GFP (corresponding to MYH6, green), ASA (far-red) and CD31 (red). *Scale bar 250 μ m.* **E)** Representative images of qPCR analysis for endothelial markers CD31 and ENDOGLIN in normoglycemic (blue) and diabetic-like condition (red). *Data are mean $\pm$ SD. $n = 4$ -5 independent biological experiments from a pool of 12 organoids/group with mean of 3 technical replicates each.*

4.4.1. Characterization of changes in the transcriptomic landscape of cardiac-like organoids exposed to normoglycemic and diabetic-like conditions

In vivo, the heart's energy metabolism undergoes rapid changes during diabetes, leading to increased fatty acid (FA) consumption and reduced glucose utilization. Many evidence suggests that this metabolic shift in the heart plays a crucial role in the development of diabetic cardiomyopathy and associated extra-cardiac conditions impacting in the function of other organs, such as the kidney (253).

To explore on the potential use of our cardiac-like organoid model to study diabetic cardiomyopathy, we assessed transcriptional changes because of the different cell culture regimes (normoglycemic and diabetic-like conditions). We selected a panel of markers related to fatty acid oxidation (FAO): Peroxisome Proliferator Activated Receptor Alpha (PPAR α); glycolysis: Hexokinase 2 (HK2) and ATP Cytrate Lyase (ACLY); mitochondrial oxidative phosphorylation system (OXPHOS): Lactate Dehydrogenase A (LDHA) and mitochondrial respiration: Carnitine Palmitoyltransferase 2 (CPT2) and Uncoupling Protein 2 (UCP2). Findings suggest an upregulation of glycolytic, OXPHOS and mitochondrial metabolic markers in diabetes-associated diseases, and a downregulation of PPAR α , the key regulator of lipid metabolism, in diabetic heart (254–258). qPCR analysis performed on cardiac-like organoids exposed to normoglycemic and diabetic-like conditions did not show any significant differences for all the analyzed genes (Fig. 24).

Diabetic heart has been also widely associated with contractile dysfunction, such as disturbance in excitation-contraction coupling. The expression of genes encoding calcium: Sarco-Endoplasmatic Reticulum Calcium ATPase (SERCA2), calcium voltage-gated channel subunit alpha 1 (CACNA1C); potassium: potassium voltage-gated channel subfamily Q member 1 (KCNQ1), Potassium Inward Rectifying channel subfamily J member 2 (KCNJ2), Potassium Voltage-Gated Channel subfamily A member 4 (KCNA4) and sodium: Sodium Voltage-Gated Channel Alpha Subunit 5 (SCNA5) can result altered (259). Through qPCR analysis, we observed a slightly upregulation of almost all cardiac ion channel markers in diabetic-like condition (Fig. 24).

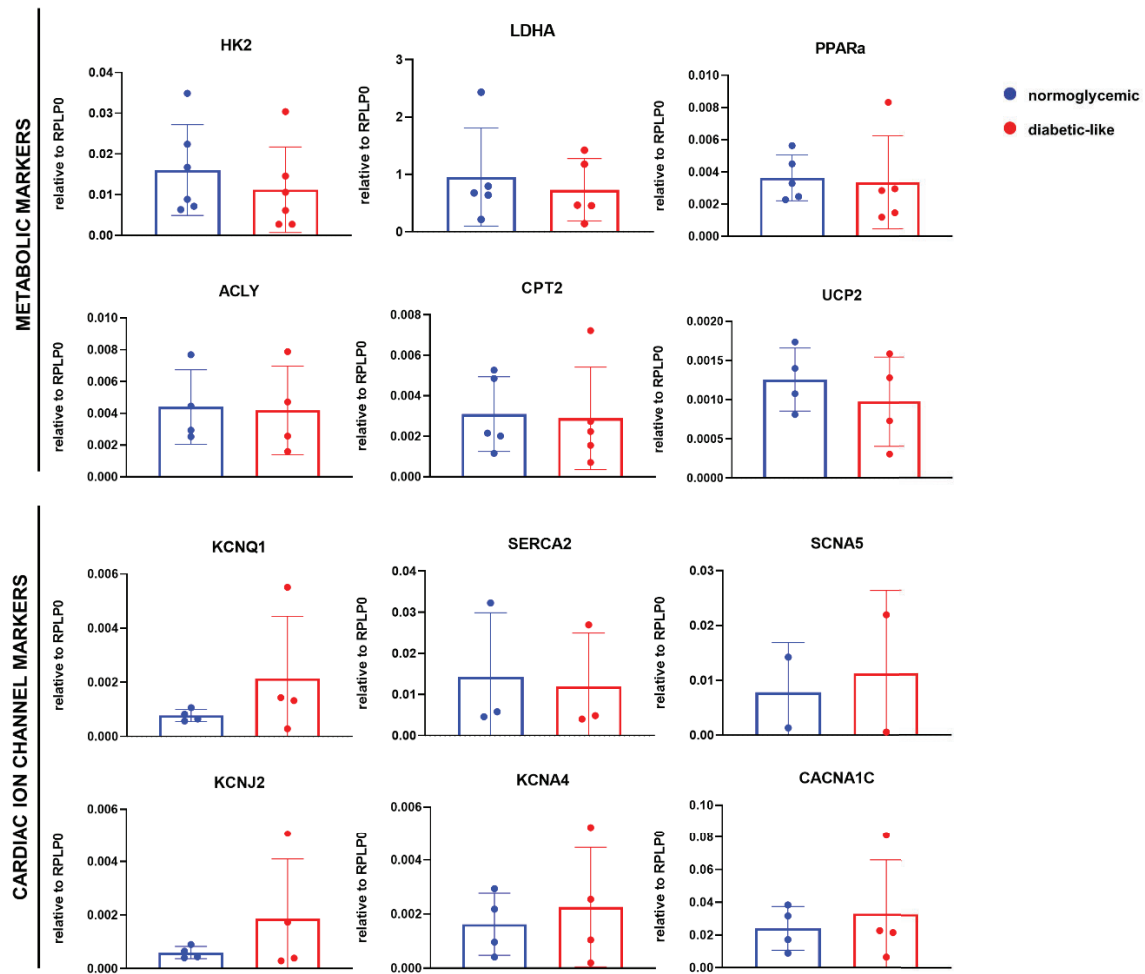


Fig. 24. Characterization of cell culture conditions mirroring normoglycemic and diabetic-like conditions in cardiac-like organoids through qPCR analysis at D30. Representative images of qPCR analysis for the indicated metabolic and cardiac ion channel markers at D30 in normoglycemic (blue) and diabetic-like (red) condition. Data are mean $\pm$ SD. n = from 2 to 5 independent biological experiments from a pool of 12 organoids/group with mean of 3 technical replicates each.

Based on our previous observations, that D60 cardiac organoids exhibit higher features of differentiation compared to that of D30 counterparts, we further exposed D70 (after one week of oscillatory regimen) cardiac-like organoids to the different cell culture regimes (normoglycemic, diabetic-like and differentiation culture conditions). In this case as well, we did not observe any significant differences in the expression of cardiac markers (*MYH6*, *SIRPA*, *TNNT2* and *TNNI3*) comparing all the different cell culture conditions, highlighting that cardiac markers do not change their levels of expression at mRNA level in front of metabolic insults. The endothelial marker *CD31* showed again an upregulation in diabetic-like condition compared to normoglycemic, as already showed at D30. The expression of cardiac ion channel markers at D70

appears to reverse compared to *D30*, showing an upregulation of normoglycemic condition compared to the diabetic-like one. Metabolic markers at *D70* did not show significant differences between normoglycemic and diabetic-like conditions (Fig. 25).

These observations parallel our findings at *D30* cardiac-like organoids (Fig. 24) pinpointing on the amenability of the procedures described in this thesis as a potential cell culture model to assess early hallmark of diabetic cardiomyopathy. Indeed, we have observed that metabolic insults did not alter cardiac marker expression, underscoring the robustness of this model. However, to comprehensively characterize our model, it is crucial to conduct a more in-depth protein-level and functional analysis rather than relying solely on mRNA levels. This approach would provide a more comprehensive understanding of the molecular changes and enable a more accurate assessment of the diabetic cardiomyopathy phenotype.

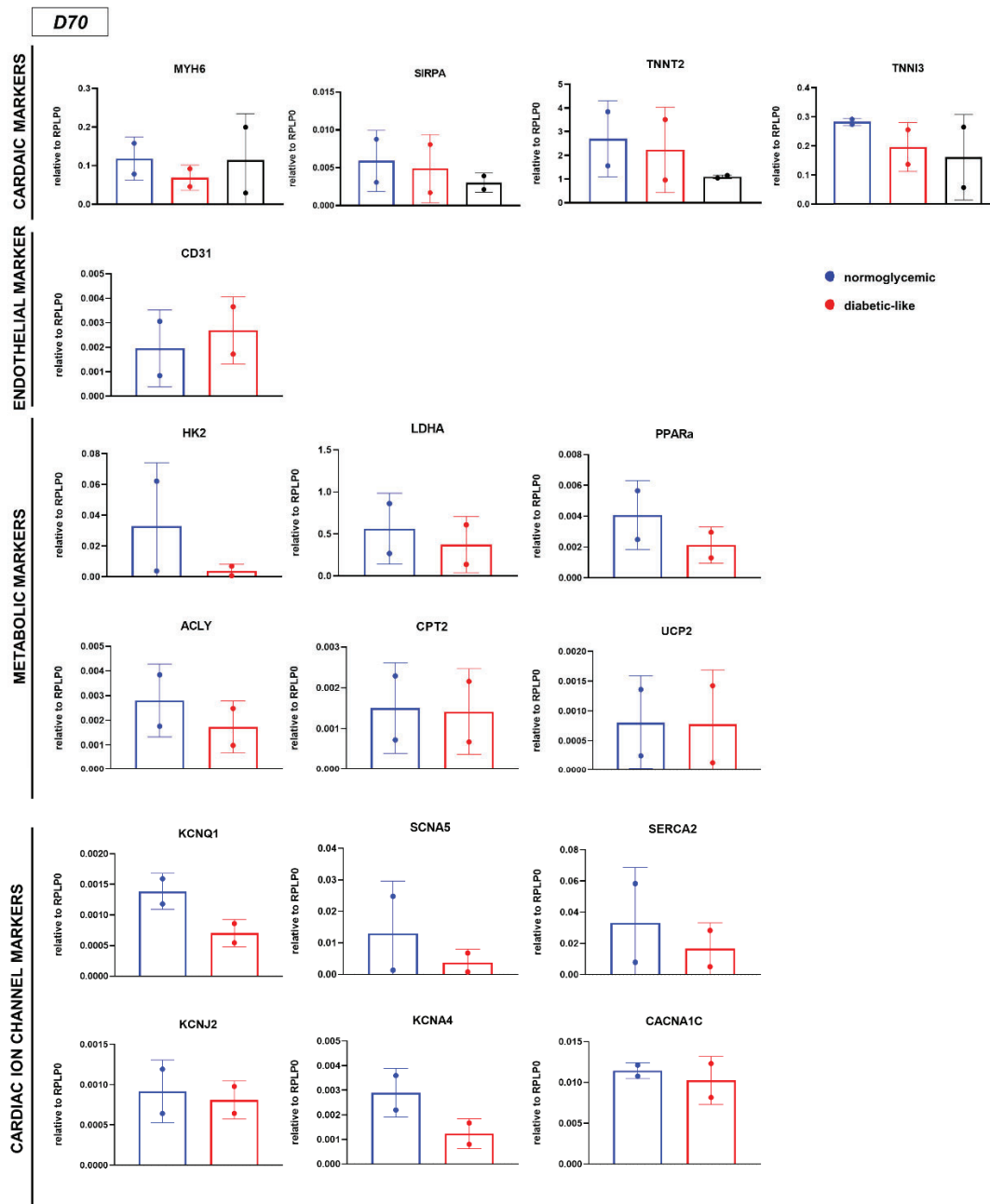


Fig. 25. Characterization of cell culture conditions mirroring normoglycemic and diabetic-like conditions in cardiac-like organoids through qPCR analysis at D70. Representative images of qPCR analysis for the indicated cardiac, metabolic, and cardiac ion channel markers at D70 in normoglycemic (blue) and diabetic-like (red) condition. Data are mean $\pm$ SD. $n = 2$ independent biological experiments from a pool of 12 organoids/group with mean of 3 technical replicates each.

4.4.2. Morphologic changes in cardiac-like organoids exposed to normoglycemic and diabetic-like conditions

Diabetes is widely recognized to have significant implications for the expression, organization, and alteration of ECM components in various organs, including the heart. The dysregulation of ECM protein accumulation and modification not only influences the mechanical characteristics of the ECM but also poses detrimental effects on cardiac function (260). The cardiac ECM is composed of a distinct network of collagen (261). Within the myocardial interstitium, fibrillar collagen types I and III are prevalent, while nonfibrillar collagen types IV and VI, as well as the glycoproteins fibronectin and laminin, are predominantly found in the myocyte basement membrane (262,263). There is a substantial body of evidence that supports the presence of myocardial fibrosis in individuals with diabetes. Diabetes-associated interstitial fibrosis is characterized by the accumulation of type I and III collagen. Moreover, this fibrotic condition affects left and right ventricles and has been described in both type 1 and type 2 diabetes (18,264). Building on this information, we conducted a comprehensive characterization of diabetic-like and normoglycemic cardiac-like organoid conditions through analysis employing optical microscopy and immunohistochemistry techniques. Bright field and H&E staining did not reveal any evident cellular morphological and tissue organization differences when comparing the extent of normoglycemic and diabetic-like culture conditions at both *D30* and *D70* (Fig. 26A). Indeed, Masson Trichrome (MT) staining showed increased collagen fiber deposition in diabetic-like cardiac-like organoids compared to normoglycemic condition at *D30*. However, these differences seemed to slightly decrease by *D70* (Fig. 26B and 26C).

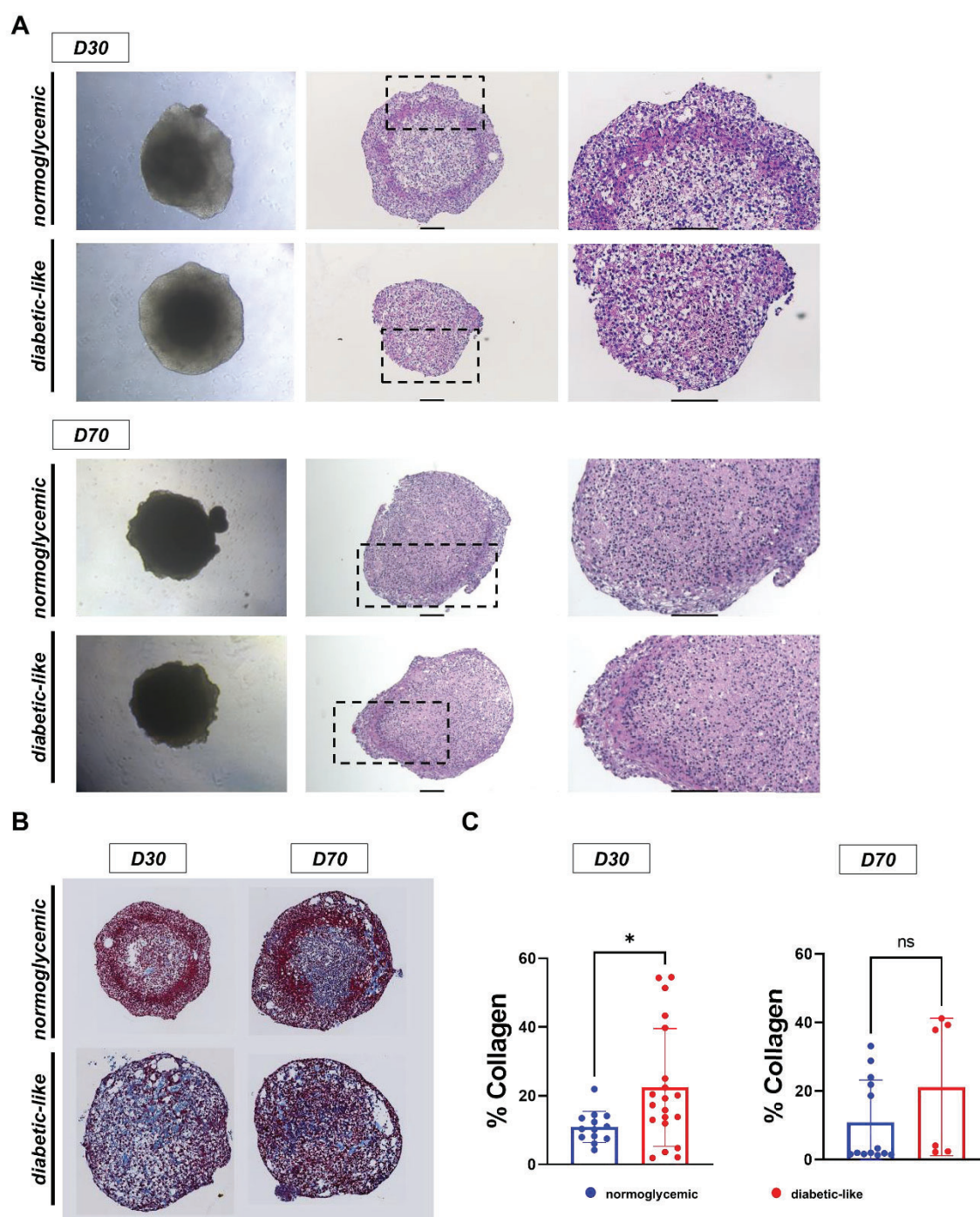


Fig. 26. Characterization of cell culture conditions mirroring a normoglycemic and diabetic-like conditions in cardiac-like organoids through optical microscopy and immunohistochemistry. A) Representative bright field and Hematoxylin and Eosin (H&E) images of normoglycemic and diabetic-like conditions in cardiac-like organoids at D30 and D70. *Scale bar 100 μ m.* **B)** Representative images of Masson Trichrome (MT) staining for the normoglycemic and diabetic-like culture conditions at D30 and D70. **C)** Corresponding quantification of collagen fibers in the entire cardiac-like organoids normoglycemic (blue) and diabetic-like (red) culture condition. Collagen was quantified as the area occupied by collagen signal normalized to the area of the full organoid. *Data are mean $\pm$ SD. At least $n = 13$ independent organoids per condition from 3 to 5 independent biological experiments. Non-parametric T-test using Mann-Whitney test. Resultant P-values are shown as ns = p value > 0.05 , and * = p value < 0.01 .*

Moreover, to test the impact of the different cell culture conditions promoting responses mirroring early hallmarks of diabetic cardiopathy, immunofluorescence analysis was conducted for the detection of ECM markers (COLLAGEN IV, FIBRONECTIN and LAMININ) together with cardiac markers (MYH6, TNNT and ASA) (Fig. 27A and 28A). The detection of ASA in paraffin sections was unsuccessful, in contrast to the whole organoids immunofluorescence analysis where we could clearly observe the sarcomere structures. As previously mentioned, no significant differences were observed between normoglycemic and diabetic-like cardiac-like organoids in cardiac marker expression. However, regarding ECM markers, the image quantification analysis corroborated the findings from MT. Interestingly, at *D30*, we noticed a higher ratio of COLLAGEN IV, LAMININ, and FIBRONECTIN in the diabetic-like condition compared to the normoglycemic condition (Fig. 27B). By *D70*, the difference between the 2 conditions decreased once again, indicating an accumulation of fibrotic tissue even in normoglycemic cardiac-like organoids (Fig. 28B). This observation aligns with the findings presented in Fig. 15B, suggesting that the progressive development of ECM components during cardiac differentiation might be contributing to this phenomenon.

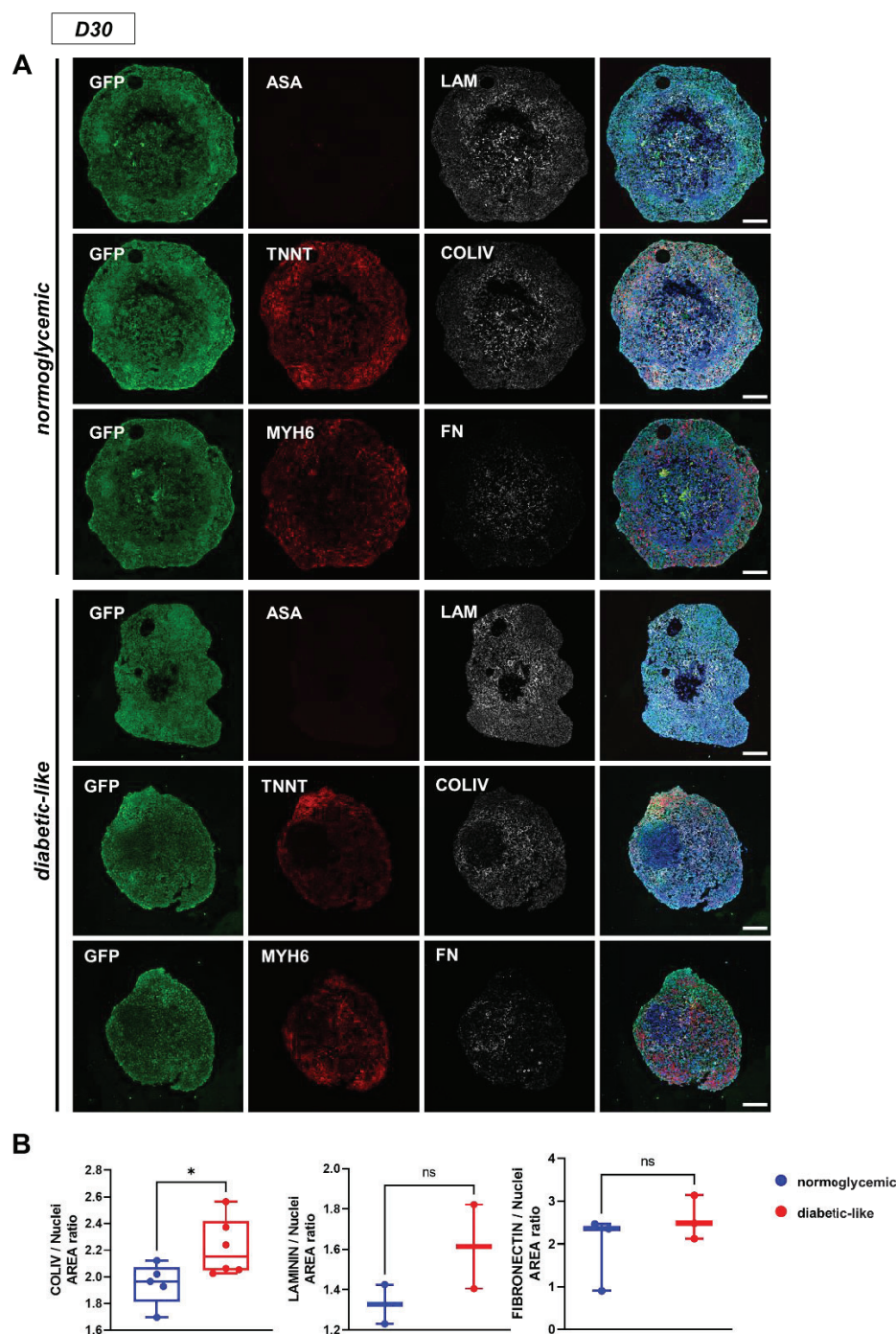


Fig. 27. Characterization of cell culture conditions mirroring a normoglycemic and diabetic-like conditions in cardiac-like organoids through immunofluorescence analysis at D30. A) Representative images of the immunofluorescence analysis performed in paraffin sections of D30 cardiac-like organoids after the exposure to normoglycemic and oscillatory glucose regimes for the detection of ECM markers LAMININ, COLLAGEN IV and FIBRONECTIN (FN) (grey), GFP (green) and cardiac markers ASA, TNNT, and MYH6 (red). Scale bar 100 μ m. **B)** Corresponding quantification of COLLAGEN IV, LAMININ and FIBRONECTIN abundance in entire cardiac-like organoid in the normoglycemic (blue) and diabetic-like (red) conditions. ECM markers were quantified as the area occupied by COLLAGEN IV, LAMININ and FIBRONECTIN signal normalized to the area for DAPI signal. Data are mean $\pm$ SD. At least $n = 2$ independent experimental replicates per condition from $n = 2$ independent biological experiments. Non-parametric T-test using Mann-Whitney test. Resultant P-values are shown as ns = p value > 0.05 , and * = p value < 0.01 .

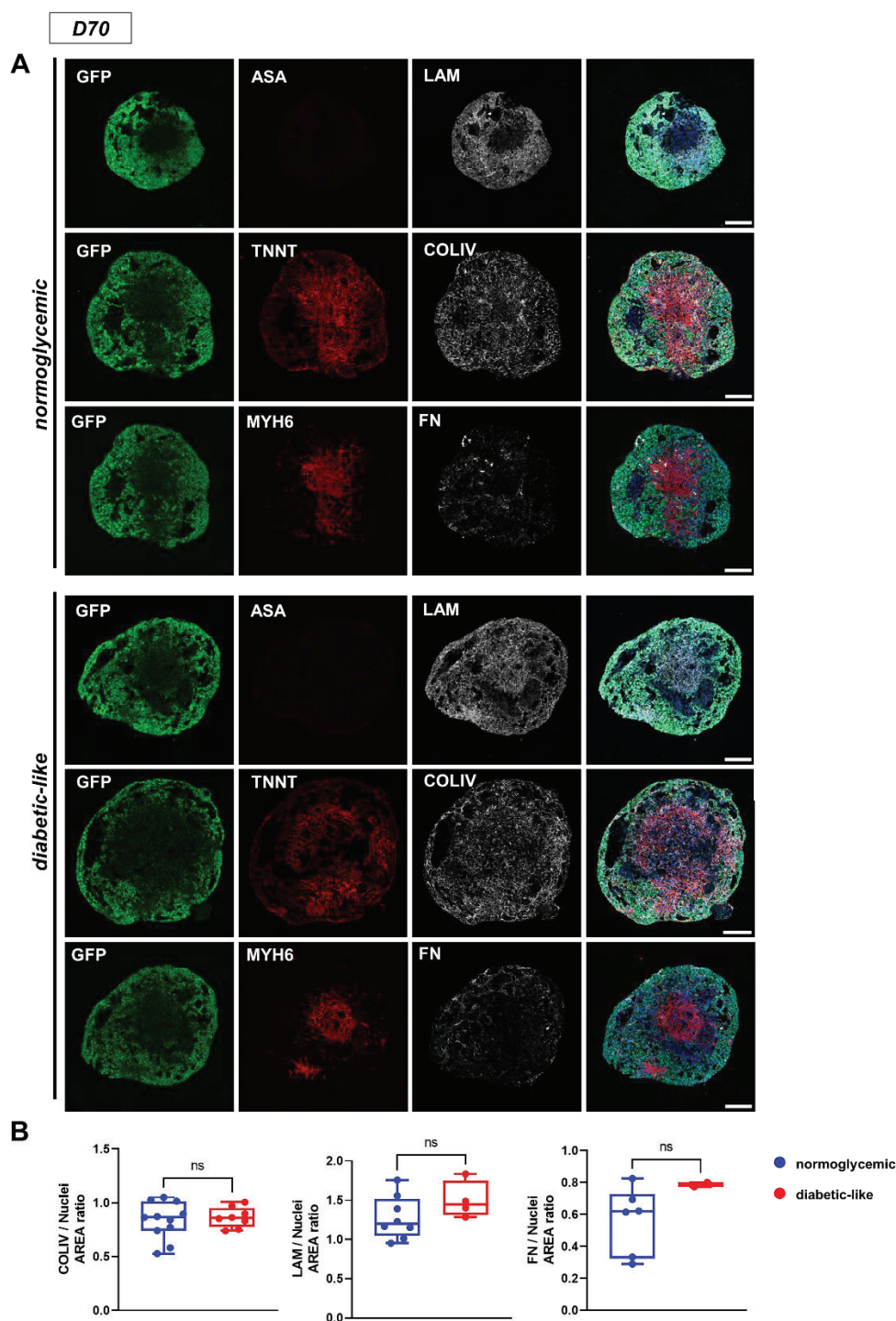


Fig. 28. Characterization of cell culture conditions mirroring a normoglycemic and diabetic-like conditions in cardiac-like organoids through immunofluorescence analysis at D70. **A)** Representative images of the immunofluorescence analysis performed in paraffin sections of D70 cardiac-like organoids after the exposure to normoglycemic and oscillatory glucose regimes for the detection of ECM markers LAMININ, COLLAGEN IV and FIBRONECTIN (FN) (grey), GFP (green) and cardiac markers ASA, TNNT and MYH6 (red). *Scale bar 100 μ m.* **D)** Corresponding quantification of COLLAGEN IV, LAMININ and FIBRONECTIN abundance in entire cardiac-like organoid in the normoglycemic (blue) and diabetic-like (red) conditions. ECM markers were quantified as the area occupied by COLLAGEN IV, LAMININ and FIBRONECTIN signal normalized to the area for DAPI signal. Data are mean $\pm$ SD. At least $n = 2$ independent experimental replicates per condition from $n = 2$ independent biological experiments. Non-parametric T-test using Mann-Whitney test. Resultant P-values are shown as ns = $pvalue > 0.05$.

4.4.3. Metabolic changes in cardiac-like organoids exposed to normoglycemic and diabetic-like conditions

As already explained, diabetes has a significant impact on fuel metabolism, with both insulin deficiency and insulin resistance leading to inefficient coupling of mitochondria and excessive production of reactive oxygen species (ROS), despite being associated with normal to high levels of oxygen consumption. Indeed, the term "*metabolic memory*" accurately describes the adverse changes resulting from hyperglycemia, even after successful regulation of blood sugar levels (265).

To augment the reliability and comprehension of our findings, we employed additional techniques to complement our data. We performed Sea horse analysis at *D30* and *D70* which allows the assessment of glycolysis and oxygen consumption, providing valuable insights into the metabolic behavior of our cardiac-like organoids. Consistent with expectations, the diabetic-like cardiac-like organoids at both time points exhibited reduced oxygen consumption rates (OCR), basal respiration, ATP production and maximal respiration compared to the normoglycemic condition, indicative of a metabolic shift previously observed in Lewis-Istracli et al. work, in which the authors modeled pregestational diabetes in self-assembling human heart organoids (41) (Fig. 29). This observation aligns with the well-documented metabolic alterations associated with diabetes and further substantiates the validity of our organoid model in recapitulating early key aspects of the disease.

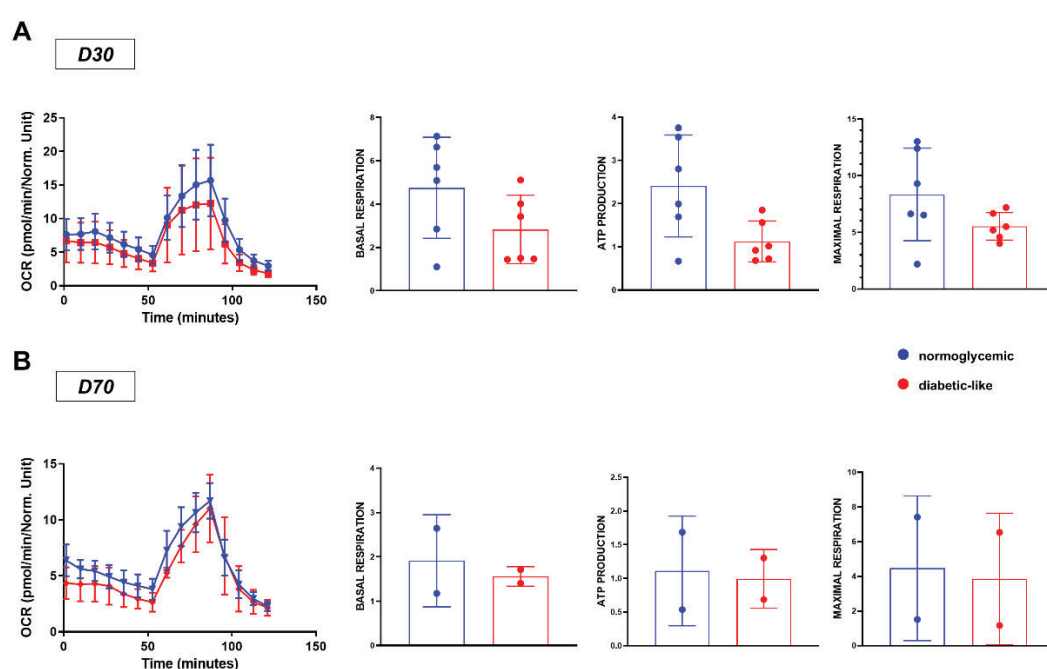


Fig. 29. Characterization of cell culture conditions mirroring a normoglycemic and diabetic-like conditions by Sea horse analysis. A) Representative image of Sea horse analysis of normoglycemic (blue) and diabetic-like condition (red) in cardiac-like organoids at *D30*. The oxygen consumption rate (OCR) data are normalized to total protein. Basal respiration, cellular ATP production, and maximal respiration are shown. *Data are mean $\pm$ SD. n= 6 independent experiments.* **B)** Representative image of Sea horse analysis of normoglycemic (blue) and diabetic-like condition (red) in cardiac-like organoids at *D70*. The oxygen consumption rate (OCR) data are normalized to total protein. Basal respiration, cellular ATP production, and maximal respiration are shown. *Data are mean $\pm$ SD. n= 2 independent experiments.*

4.4.4. Ultrastructural changes in cardiac-like organoids exposed to normoglycemic and diabetic-like conditions

To gain insights into the intricate alterations occurring at the subcellular level, TEM analysis was employed at *D30* in normoglycemic and diabetic-like cardiac-like organoids. We aimed to elucidate the effects of glucose fluctuations on the microstructural integrity of cardiac-like organoids. This investigation provides a deeper understanding of the cellular response to normoglycemic and diabetic-like environments and sheds light on the potential impact of glucose dynamics on cardiac-like tissue architecture. Semithin sections of normoglycemic and diabetic-like condition did not yield substantial information (Fig. 30A). Indeed, TEM imaging revealed that a diabetic-like microenvironment results in a reduced number of mitochondria and a higher number of lipid droplets compared to a control counterpart (normoglycemic exposure), suggesting dysfunctional lipid metabolism and a shift towards a glycolytic phenotype (Fig. 30B).

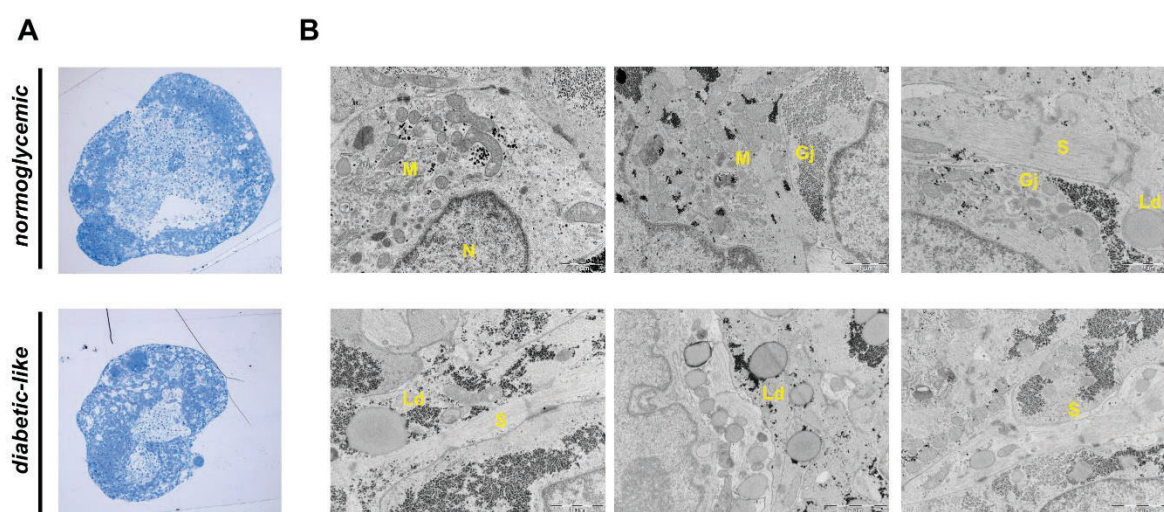


Fig. 30. Characterization of cell culture conditions mirroring a normoglycemic and diabetic-like conditions by TEM. A) Semithin sections of normoglycemic and diabetic-like condition cardiac-like organoids at *D30*. **B)** Representative images of TEM analysis showing mitochondria (M), sarcomeres (S), lipid droplets (Ld), nucleus (N) and gap junction (Gj) in normoglycemic and diabetic-like conditions of cardiac-like organoids at *D30*. Scale bar 1 μ m.

4.4.5. Characterization of calcium transients in cardiac-like organoids exposed to normoglycemic and diabetic-like conditions

Diabetic patients show an increased occurrence of cardiac arrhythmias, including ventricular fibrillation. Electrocardiogram (ECG) readings reveal significant alterations primarily linked to ventricular repolarization. These alterations include higher heart rates, reduced ECG potential amplitudes, and a greater number of T-wave inversions compared to non-diabetic individuals. Additionally, patients with diabetic cardiomyopathy often exhibit impaired contraction and abnormal ECG patterns (259). In this regard, in collaboration with the Hospital General Universitario Gregorio Marañón in Madrid, a functional assay was conducted on cardiac-like organoids at *D30* and *D70*, comparing normoglycemic and diabetic-like conditions. At both *D30* and *D70*, cardiac-like organoids under normoglycemic conditions displayed regular and coordinated calcium transients, indicative of well-functioning excitation-contraction coupling. In contrast, exposure to diabetic-like regimes resulted in aberrant calcium handling patterns, characterized by irregular calcium transients, and increased frequency. Indeed, the duration of calcium transients (CaTD50 and CaTD70) was significantly prolonged in cardiac-like organoids subjected to diabetic-like condition, indicating impaired calcium reuptake mechanisms (as explained in 4.3.5 section). At *D30* cardiac-like organoids responded to flecainide and exhibited an antiarrhythmic effect with longer CaT50 and CaT70 durations. The stimulated results found a better match for capturing external stimuli in the case of the normoglycemic than the diabetic-like condition in the bivariate statistical tests performed (Fig. 31A). In the case of *D70*, the flecainide effect was stronger in the diabetic-like than normoglycemic condition. The stimulated rhythms from the external source produced a better synchronization in the normoglycemic than in the diabetic-like condition for both the CaT50 and the CaT70 durations. The diabetic-like condition did not capture the external stimuli, as the normoglycemic did, notice the downslope tendency in both left bars of the CaT50 and CaT70 panels (Fig. 31B). To sum up, normoglycemic condition at both *D30* and *D70* exhibited diminished CaTD, indicating enhanced calcium regulation and faster

response times, suggesting better functional characteristics compared to diabetic-like condition.

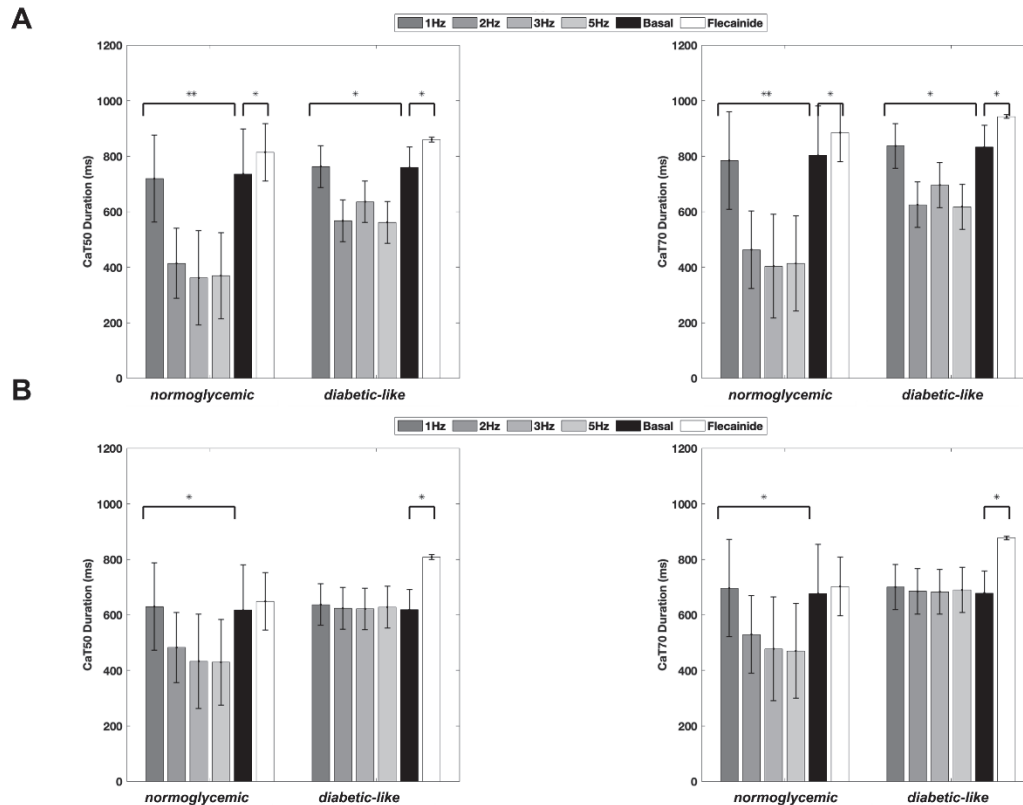


Fig. 31. Characterization of calcium transients in cardiac-like organoids exposed to normoglycemic and diabetic-like conditions. A) Optical mapping calcium transient CaT50 (left) and CaT70 (right) durations for the normoglycemic and diabetic-like conditions at *D30*. Optical mapping images were acquired following the same protocol as depicted in Fig. 20 and the same statistical tests were performed. (*) p-value <0.05; (**) p-value<0.001. *Data are mean of n= 24 organoids from n= 1 independent experiment.* **B)** Optical mapping calcium transient CaT50 (left) and CaT70 (right) durations for the for the normoglycemic and diabetic-like conditions at *D70*. The same optical mapping stimulation protocol and statistical analysis were performed as the one for *D30* in Fig. 30A. (*) p-value <0.05; (**) p-value<0.001. *Data are mean of n= 24 organoids from n= 1 independent experiment.*

Chapter III: Exploiting the heart and kidney ECM as a natural biomaterial to enhance organoid differentiation

4.5. Decellularization of porcine and human kidney and porcine heart tissues to generate acellular scaffolds for ulterior applications in organoid engineering

Despite the significant advancements in kidney and heart differentiation from hPSCs, the current state of hPSC-derived organoids still lags the complexity and functionality of mature tissue(s). The use of novel bioengineering approaches can help mimicking the hierarchical architecture of an organ *in vitro*. Within the last decades tissue decellularized ECM (dECM) has been used as a biocompatible scaffold or as a primary source for the generation of dECM-derived hydrogels. To date, the use of acellular ECM as a biomaterial promoting differentiation of hPSCs has been reported in several studies (50,52,54,55). Indeed, the host laboratory generated human cardiac grafts through the combination of hPSCs-CLCs together with dECM-derived from human myocardium further demonstrating on the impact of cardiac dECM promoting hPSCs-CLCs differentiation and function in front of other ECM matrices such as Matrigel (56).

In this regard, in this thesis we worked towards the generation of novel approaches exploiting the inherent characteristics of acellular ECM-derived biomaterials promoting hPSCs differentiation. It is well known that ECM-derived biomaterials, namely hydrogels, possess unique features that make them highly suitable for supporting cellular growth, migration, and differentiation. To that, we first defined new procedures to establish decellularization conditions allowing for the generation of porcine and human kidney and cardiac dECM-derived hydrogels.

As already mentioned, decellularization techniques strive to eliminate cellular components from tissues or organs while maintaining the original ECM composition and architectural integrity. With respect to the techniques used to decellularized complex organs, such as kidney and heart, perfusion through the vasculature and immersion/agitation are nowadays the most used (46). In this thesis, and based on tissue availability, kidney- and heart-derived tissues were decellularized by immersion/agitation. To this, small pieces of tissues were first submerged into decellularization solutions (sodium dodecyl sulfate, SDS) while being subjected to agitation. Thus, decellularization agents permeated the tissue *via* diffusion, thereby determining the duration of the protocol based on the initial thickness of the tissue and

cell density. For kidney and heart, approximately, 1 cm² of the kidney cortex and heart ventriculi were minced by dissection, respectively. After washing in detergent solutions (SDS and milliQ H₂O) the DNA content was determined. The success of the different decellularization experiments was evaluated by the quantification of total genomic DNA (Fig. 32B and 32D). Our results showed ECM scaffolds containing less than 50 ng/mg DNA of initial wet tissue, thus highlighting that the procedure enables for the generation of biological-derived acellular scaffolds that in turn preserve some of the major ECM proteins while also maintaining tissue macrostructure as detailed below.

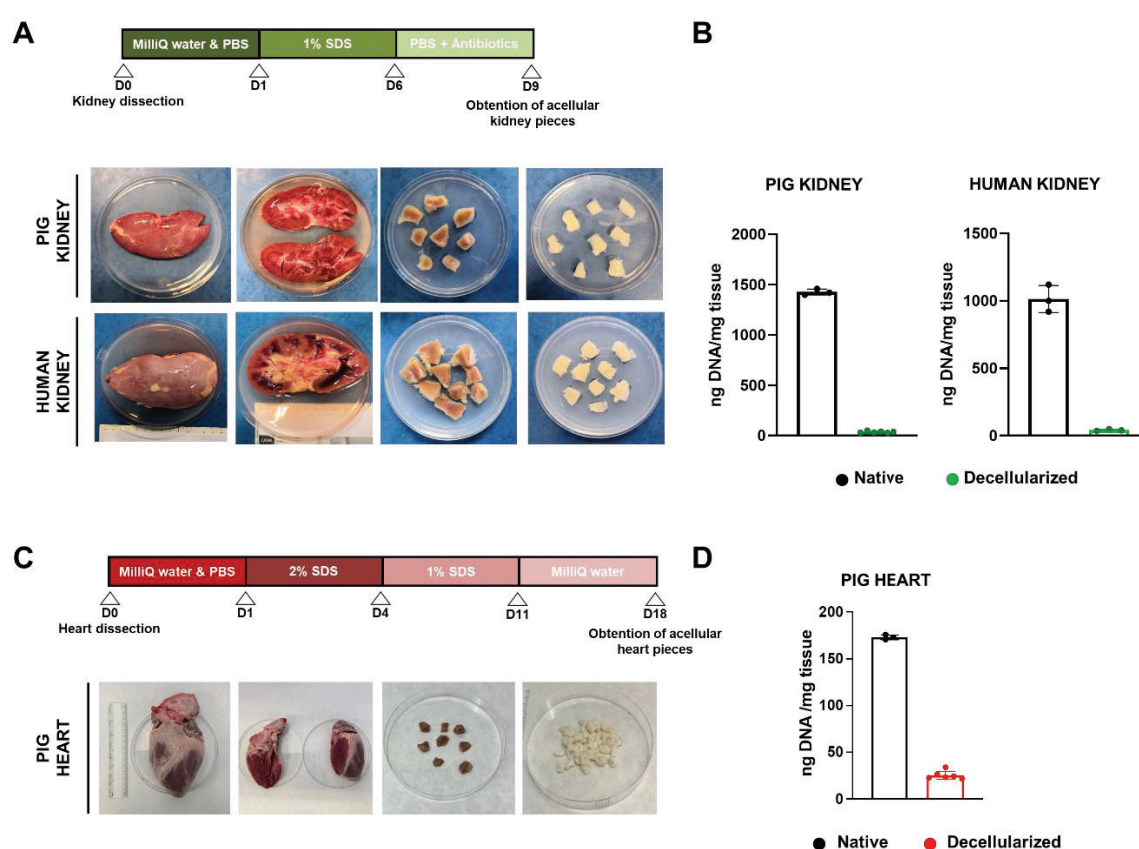


Fig. 32. Generation of acellular scaffolds from porcine and human tissues, namely kidney and heart. **A)** Representative images of porcine and human kidneys during immersion-agitation protocol using enzymatic digestion based on the use of SDS detergent. **B)** Respective DNA quantification of porcine and human kidney comparing native versus the resulting decellularized tissues. DNA quantification is represented as ng/mg initial wet tissue. *Data are mean $\pm$ SD. At least $n=3$ technical replicates from 2 independent experiments of decellularization.* **C)** Representative images of porcine heart during immersion-agitation protocol using enzymatic digestion based on the use of SDS detergent. **D)** Respective DNA quantification of porcine heart comparing native versus the resulting decellularized tissues. DNA quantification is represented as ng/mg initial wet tissue. *Data are mean $\pm$ SD. At least $n=3$ technical replicates from 2 independent experiments of decellularization.*

4.5.1. Morphological characterization of kidney and heart acellular scaffolds

To determine tissue macrostructure prior and after tissue(s) decellularization, histological and immunocytochemistry analysis were performed. Both approaches demonstrated the absence of cellular material and the integrity of major compartments successively the process. Our study utilized various staining techniques to assess the characteristics of the ECM in both native and decellularized tissue. H&E staining confirmed the integrity of the ECM. Hematoxylin, a basic dye, creates a blue-purple contrast on basophilic structures, particularly those that harbour nucleic acid moieties like chromatin, ribosomes, and RNA-rich cytoplasmic regions. An acidic eosin, on the other hand, provides a counterstain for the basic elements, such as red blood cells (RBCs), cytoplasm, muscle, and collagen, exhibiting shades of pink, orange, and red with varying intensities. Additionally, MT staining was employed to demonstrate the preservation of collagenous matrix proteins, indicated by a blue colour, while the loss of cytoplasm was visualized as a magenta hue. This staining uses 3 dyes to selectively stain muscle, collagen fibers, fibrin and erythrocytes. In both kidney and heart decellularized tissues, at the end of the process shades of pink (H&E) and blue (MT) were observed, indicating the loss of cellular structures and the preservation of extracellular matrix (collagen). Verhoeff's elastic (EVG) staining reveals the undamaged elastic laminae of the blood vessels, which appeared pink and brown the elastic fibers in the native heart tissue. Finally, periodic acid-Schiff (PAS) is a staining which chemically converts sugars in glycogen into a magenta-coloured product. In the kidney, the presence of polysaccharides is displayed on the brush borders of proximal tubules and the basal membranes of tubuli and glomeruli in both native and decellularized tissue. Instead, in the heart the magenta shows the presence of Purkinje fibers, specialized cardiac muscle cells that conduct electrical impulses that allow coordinated contraction of cardiac muscle. All these stainings showed the loss of nuclei in decellularized samples compared to native tissue (Fig. 33A,34A,35A). Using immunohistochemistry, we also aimed to study the detection of specific renal and cardiac makers prior and after the decellularization process. For the kidney, we made use of *Lotus Tetraglobus Lectin* (LTL) to label proximal tubular cells and PODOCALYXIN (PODXL) to further detect podocytes. These markers were used together with antibodies of the basement membrane proteins COLLAGEN IV (COLIV) and LAMININ (LAM) to further highlight the presence of ECM proteins together with

major renal markers (Fig. 33B and 34B). For the heart, we detected MYH6 as specific cardiac marker together with COLAGEN I (COL I), COL IV, LAM and FIBRONECTIN (FN) for the maintenance of extracellular structures (Fig. 35B).

Furthermore, in both organs we took advantage of the Alpha-Gal xeno-antigen marker (A-GAL) to ascertain the reduction of the alpha-galactosidase epitopes in the porcine dECM scaffolds in comparison with native tissues. Alpha-Gal epitopes is present in animal tissues and is known as one of the key xeno-antigens triggering xeno-rejection (266). Interestingly, A-GAL xeno-antigen was undetectable in porcine kidney and heart dECMs in comparison to native tissues.

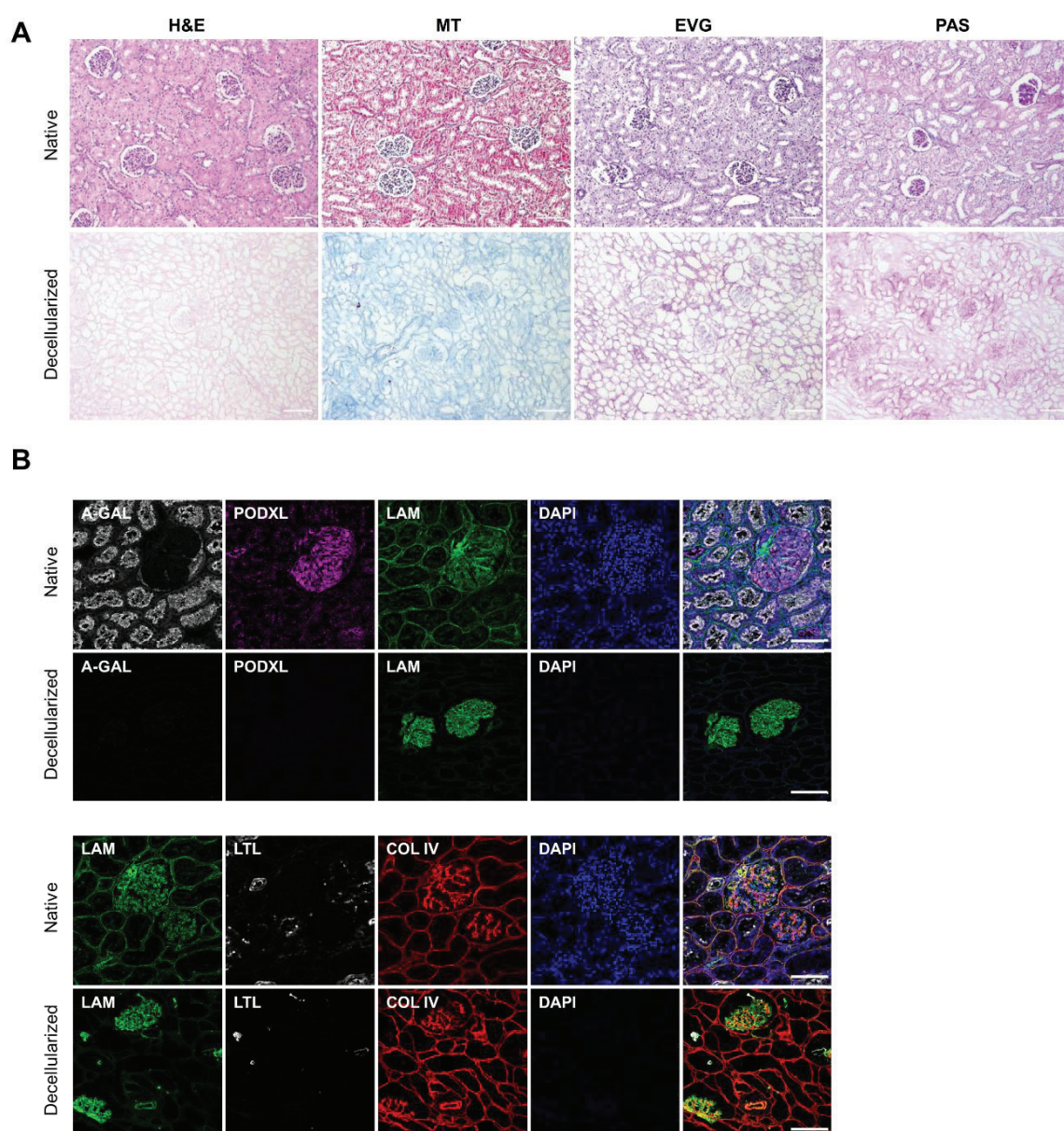


Fig. 33. Morphological characterization of porcine kidney acellular scaffolds. **A)** H&E, MT, EVG and PAS stainings in porcine kidney native tissue compared with decellularized tissue. *Scale bar 100 μ m.* **B)** Representative images of the immunofluorescence analysis performed in paraffin sections of porcine kidney native tissue compared with decellularized tissue for the detection of specific renal marker for proximal-like tubular cells (LTL, grey), podocyte-like cells (PODXL, magenta). ECM markers: LAMININ (LAM, green), COLLAGEN IV (COL IV, red) and Alpha-Gal (A-GAL, grey). *Scale bar 100 μ m.* Representative images from at least $n=2$ independent experiments of decellularization.

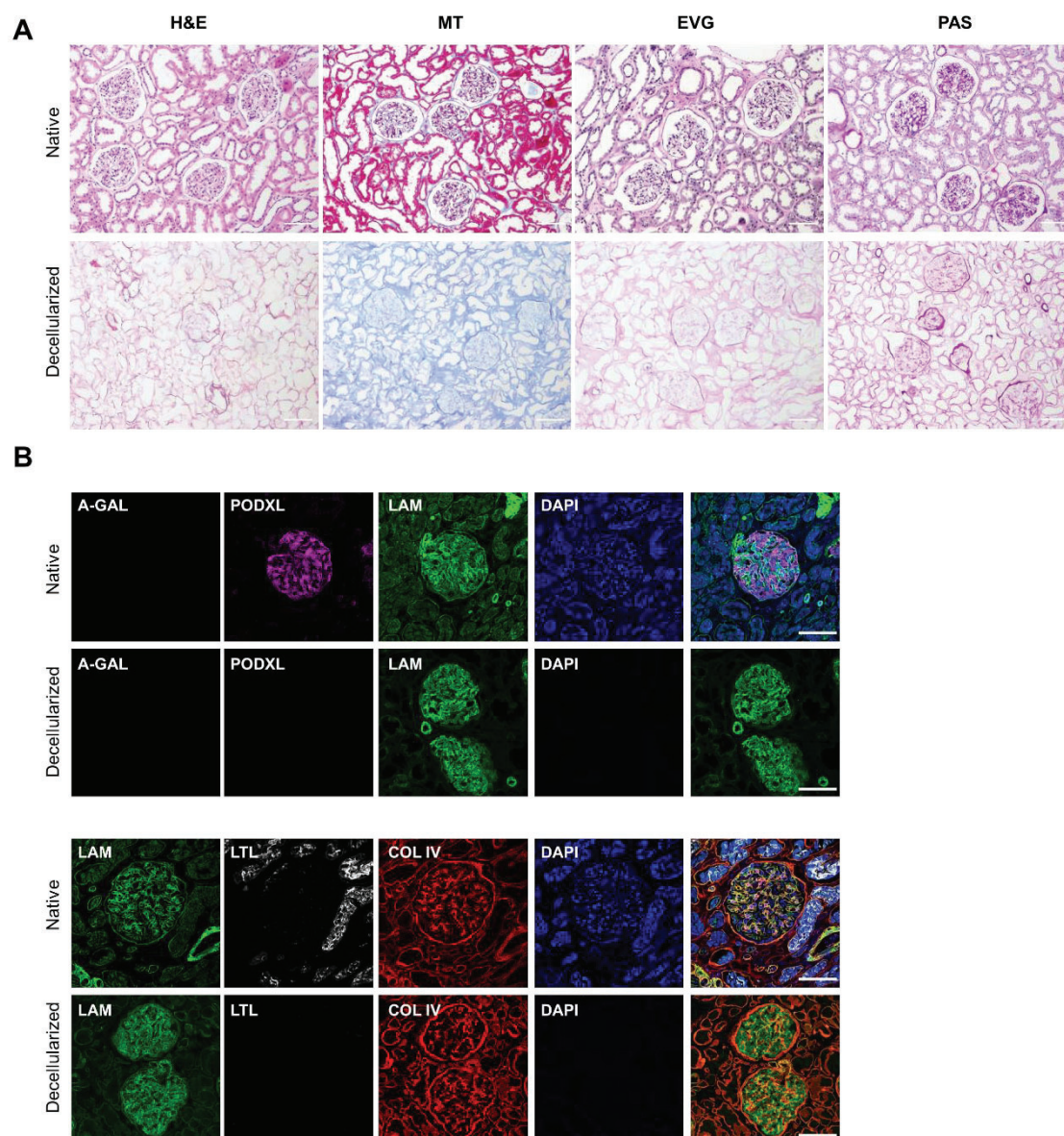


Fig. 34. Morphological characterization of human kidney acellular scaffolds. **A)** H&E, MT, EVG and PAS stainings in human kidney native tissue compared with decellularized tissue. *Scale bar 100 μ m.* **B)** Representative images of the immunofluorescence analysis performed in paraffin sections of human kidney native tissue compared with decellularized tissue for the detection of specific renal marker for proximal-like tubular cells (LTL, grey), podocyte-like cells (PODXL, magenta). ECM markers: LAMININ (LAM, green), COLLAGEN IV (COL IV, red) and Alpha-Gal (A-GAL, grey). *Scale bar 100 μ m.* Representative images from at least $n=2$ independent experiments of decellularization.

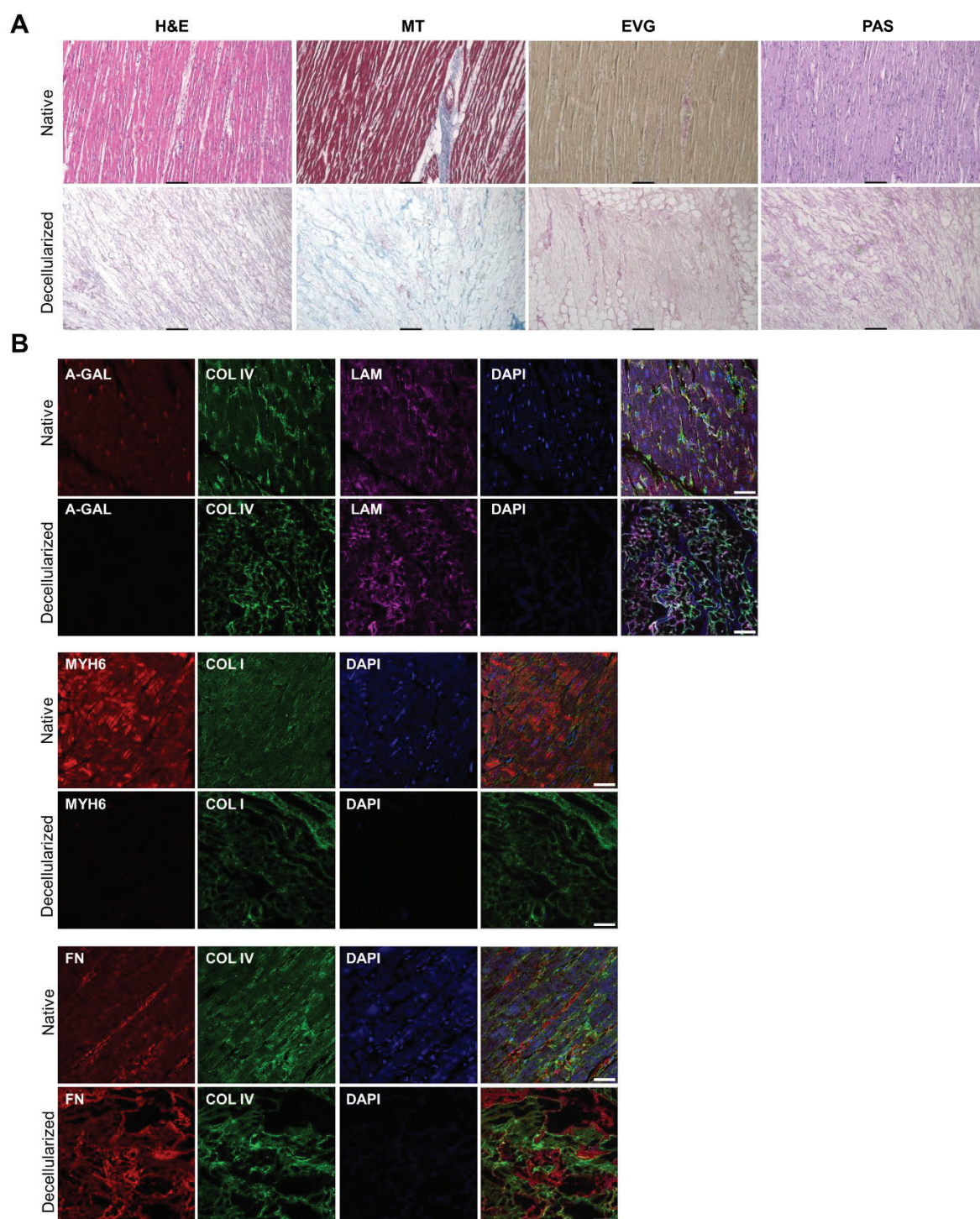


Fig. 35. Morphological characterization of heart acellular scaffolds. A) H&E, MT, EVG and PAS staining in porcine heart native tissue compared with decellularized tissue. *Scale bar 100 μ m.* **B)** Representative images of the immunofluorescence analysis performed in paraffin sections of human kidney native tissue compared with decellularized tissue for the detection of specific cardiac marker MYH6 (red), ECM markers: LAMININ (LAM, magenta), COLLAGEN I (COL I, green), COLLAGEN IV (COL IV, green), FIBRONECTIN (FN, red) and Alpha-Gal (A-GAL, grey). *Scale bar 100 μ m.* Representative images from at least $n=2$ independent experiments of decellularization.

4.5.2. Generation of hydrogels from kidney and heart acellular scaffolds

To harness the potential of dECM as a natural substrate facilitating organoid differentiation, we successfully derived hydrogels from kidney and heart acellular tissues. The generation of the different hydrogels consisted in a first step of freeze-drying process. Briefly, acellular scaffolds were kept at -80°C overnight and further lyophilized for 48 h. The resulting samples were powdered and digested with pepsin for 48h under constant agitation. After homogenization, solutions were concentrated, and stocks were prepared using 10X PBS to maintain the osmotic pressure and pH was adjusted to neutral (Fig. 36).

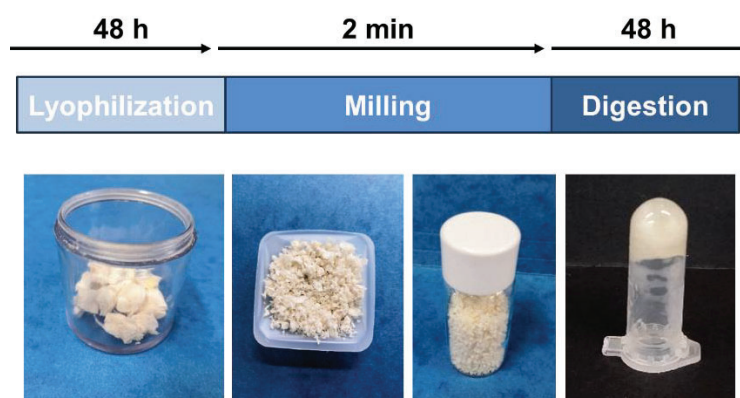


Figure 36. Generation of hydrogels from kidney and heart acellular scaffolds. Representative images for the generation of kidney and heart dECM hydrogels. Decellularized tissues are frozen at -80°C overnight followed by lyophilization for 48h. Dried dECM pieces are milled into powder that is further digested using an acid pepsin solution for 48h. After digestion, salt content is neutralized using 10X PBS. *Representative images from at least $n=2$ independent experiments of both kidney and heart decellularization.*

4.5.3. Encapsulation of hPSCs-kidney progenitor cells within renal dECM-derived hydrogels as a new procedure to generate kidney organoids

Although significant progress has been made in the field of kidney organoids regarding the description of procedures allowing for the generation of nephron-like stage organoids for applications in precision medicine and developmental biology, further strategies are still needed to improve their proper maturation and function (39,171–173). An amenable approach entails studying kidney-derived ECM materials as a biological source promoting differentiation. In this regard, pioneering work from the laboratory of Orlando already demonstrated that discarded human kidney can serve as a viable source of renal scaffolds, and their utilization in tissue engineering

applications could hold greater clinical relevance compared to kidneys obtained from animals (267). Here, we explored the role of porcine renal dECM-derived hydrogel as a new biomaterial sustaining hPSCs differentiation into kidney organoids. To enhance the stability of the construct, a combination of porcine renal dECM-derived hydrogel together with well-established biomaterials such as gelatin and fibrin was assayed. The resulting hydrogel(s) were merged with hPSCs-kidney progenitor cells at day *D4* of differentiation based on previous results of the host laboratory demonstrating the expression of nephron progenitor markers at this stage of differentiation (39). Kidney organoids were generated from hPSC by initiating the formation of the primitive streak on *D0* using CHIR. Subsequently, hPSCs were directed towards intermediate mesoderm (IM). On *D4*, hPSC-derived IM-like cells were enzymatically digested and single cell suspensions were further aggregated as spheroids by spin centrifugation which would eventually give rise to renal vesicle (RV)-like structures starting from *D9*. To that the hydrogel solution was prepared just before the encapsulation of *D4* hPSCs-NPC-like cells. *D4* hPSCs-NPC-like cells were detached in single cells and counted to obtain a final cell density ranging from 15 to 25 million cells/mL. The appropriate volume of cell suspension was centrifugated and the pellet mixed with the hydrogel solution to achieve a final concentration of 5 mg/mL fibrinogen, 5% gelatin, 2.5 mM CaCl₂, and 0.1% transglutaminase, with or without 15 wt/v% dECM. Then, bioink drops containing hPSCs-IM-like cells were seeded in trans-well inserts and cross-linked with 10 U/mL thrombin. hPSCs-kidney organoids constructs were maintained in hPSCs-kidney differentiation media until *D20* (Fig. 37A). Bright field images and H&E staining showed the formation of RV-like structures at *D12* and more complex nephron-like structures at *D20* in all the experimental conditions (Fig. 37B).

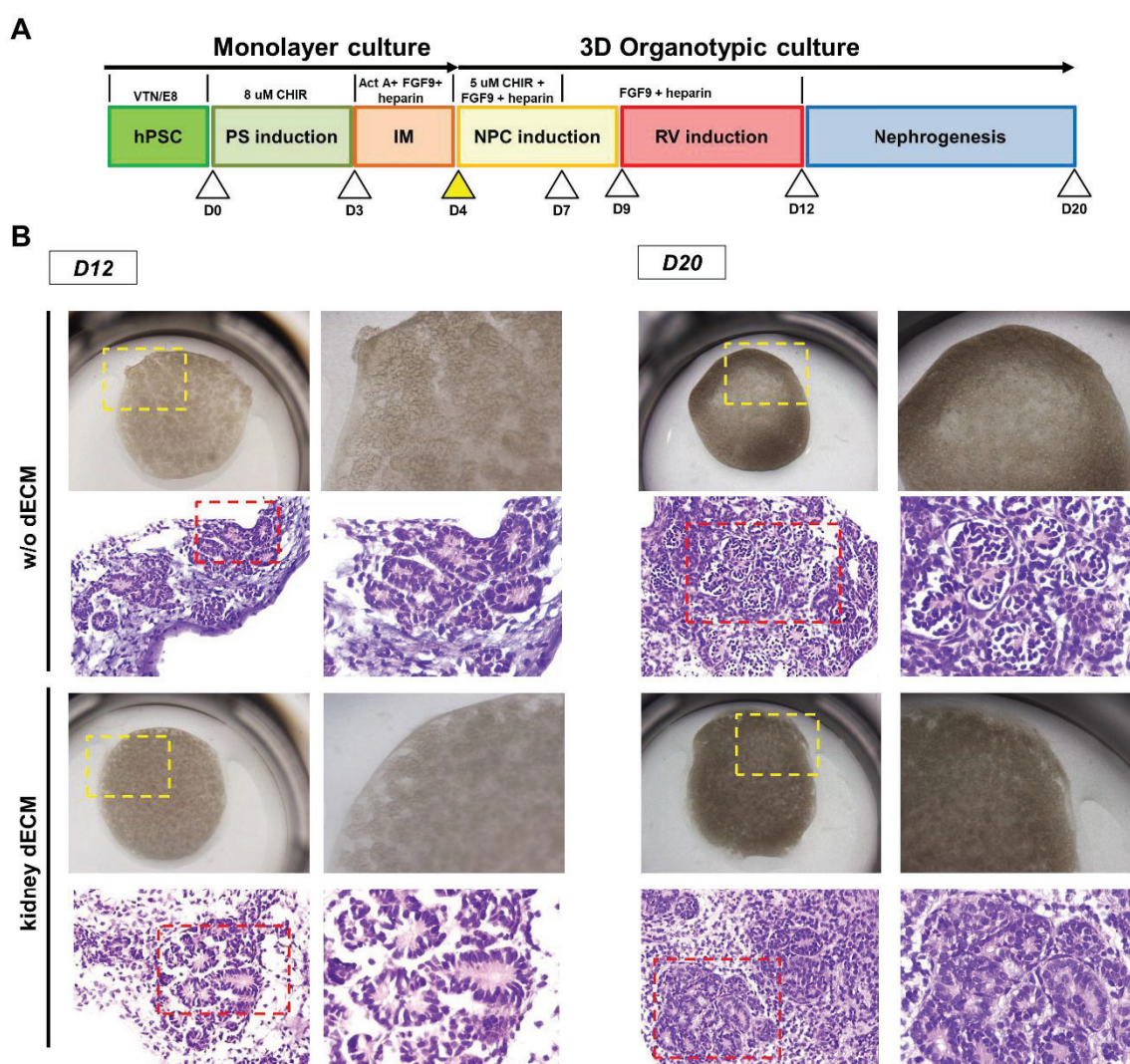


Fig. 37. Encapsulation of hPSCs-kidney progenitor cells within renal dECM-derived hydrogels. **A)** Experimental scheme for the differentiation of kidney organoids from hPSCs. *D4* hPSCs-IM-like cells are mixed with renal dECM-derived hydrogel solution, fibrinogen and gelatin and maintained in culture until *D20* with and without renal dECM-derived hydrogel. **B)** Representative bright field images and H&E staining of hPSC-kidney organoids construct with and without renal dECM-derived hydrogel at *D12* and *D20*. Representative images from *n=1* independent biological experiment.

4.5.4. Analysis on the impact of renal dECM-derived hydrogels for the derivation of hPSCs-kidney organoids by means of immunofluorescence and qPCR analysis

To investigate the role of renal dECM-derived hydrogels on the efficient generation of hPSCs-kidney differentiation, further analysis was performed at *D20*. Immunofluorescence analysis was performed to assess the detection of markers of different segments of the nephron including markers to detect proximal-like tubular cells (LTL), markers for podocyte-like cells as NPHS2 and NPHS1. Moreover, the

detection of markers for the distal compartment of the nephron were also used (E-cadherin) as well as endothelial markers including CD34 and CD31 (Fig. 38). Overall, the results highlighted no significant differences in the detection of the different markers when comparing the different culture conditions for immunofluorescence analysis.

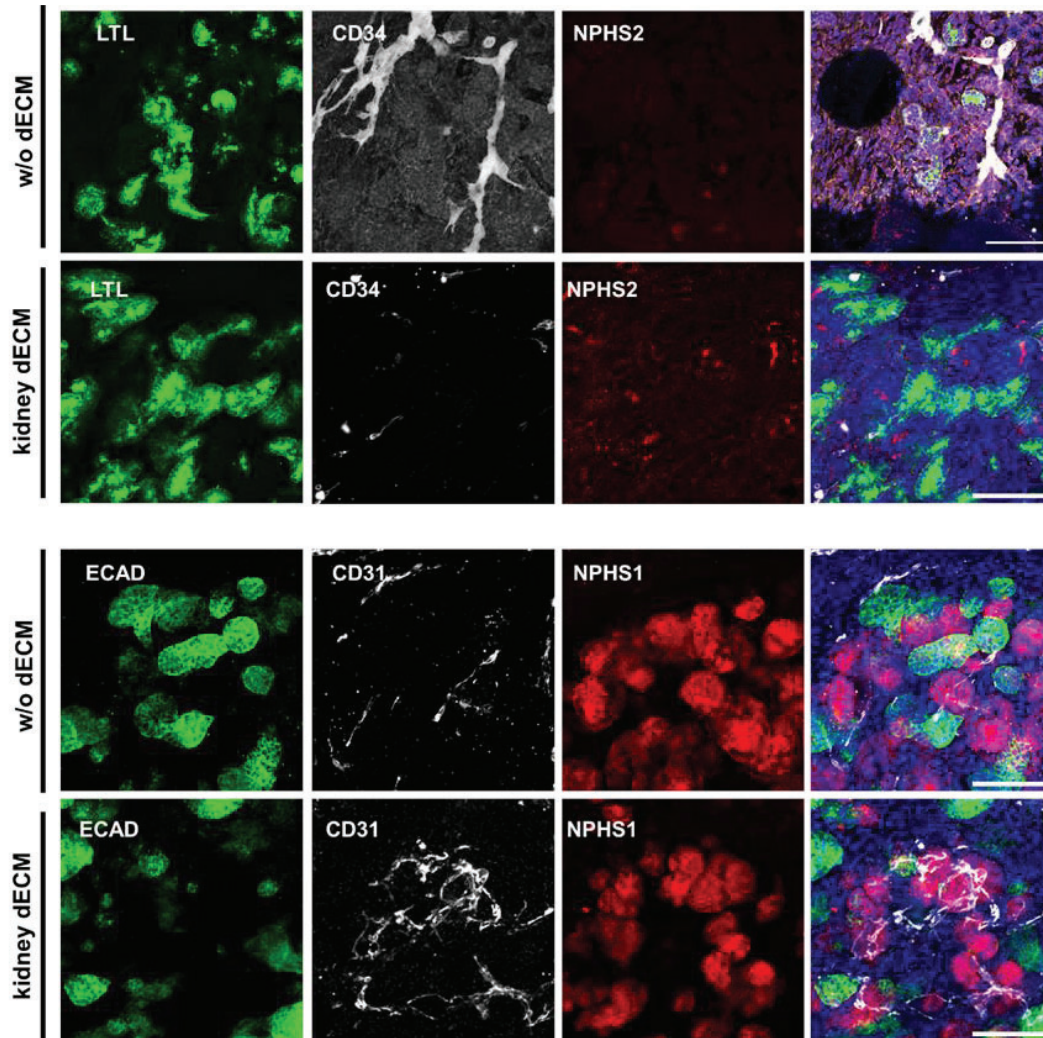


Fig. 38. Characterization of encapsulated hPSCs-kidney progenitor cells within renal dECM-derived hydrogels through immunofluorescence analysis. Representative images of the immunofluorescence analysis performed in *D20* hPSC-kidney organoids after the encapsulation of *D4* hPSC-IM-like cells without (w/o dECM) and with renal dECM-derived hydrogels (dECM). On top, nephron-like structures positively labelled with LTL (green) for proximal tubular-like cells, the podocyte marker NPHS2 (red) and the endothelial marker CD34 (grey); on bottom, nephron-like structures positively labelled with the distal compartment of nephron marker E-cadherin (ECAD; green), the endothelial marker CD31 (grey) and the glomerular marker NPHS1 (red). Scale bar 100 μm . Representative images from $n=1$ independent biological experiment.

As expected, qPCR at *D20* showed higher mRNA expression levels for collagens *COL1A1*, *COL3A1*, *COL4A1* in *D20* hPSC-kidney organoids generated from *D4* hPSC-IM-like cells encapsulated with renal dECM-derived hydrogels compared to control counterparts. Similar results were observed when analyzing the mRNA levels of nephron progenitor markers (*SIX2*, *PAX2*), ureteric bud progenitor markers (*GATA3*, *GDNF*) and markers of the glomerular compartment (*NPHS1*, *PODXL*) (Fig. 39). Overall, our results indicate an enhancement of differentiation in hPSCs-kidney organoids generated from *D4* hPSC-IM-like cells encapsulated with renal dECM-derived hydrogels in front of control cell culture conditions.

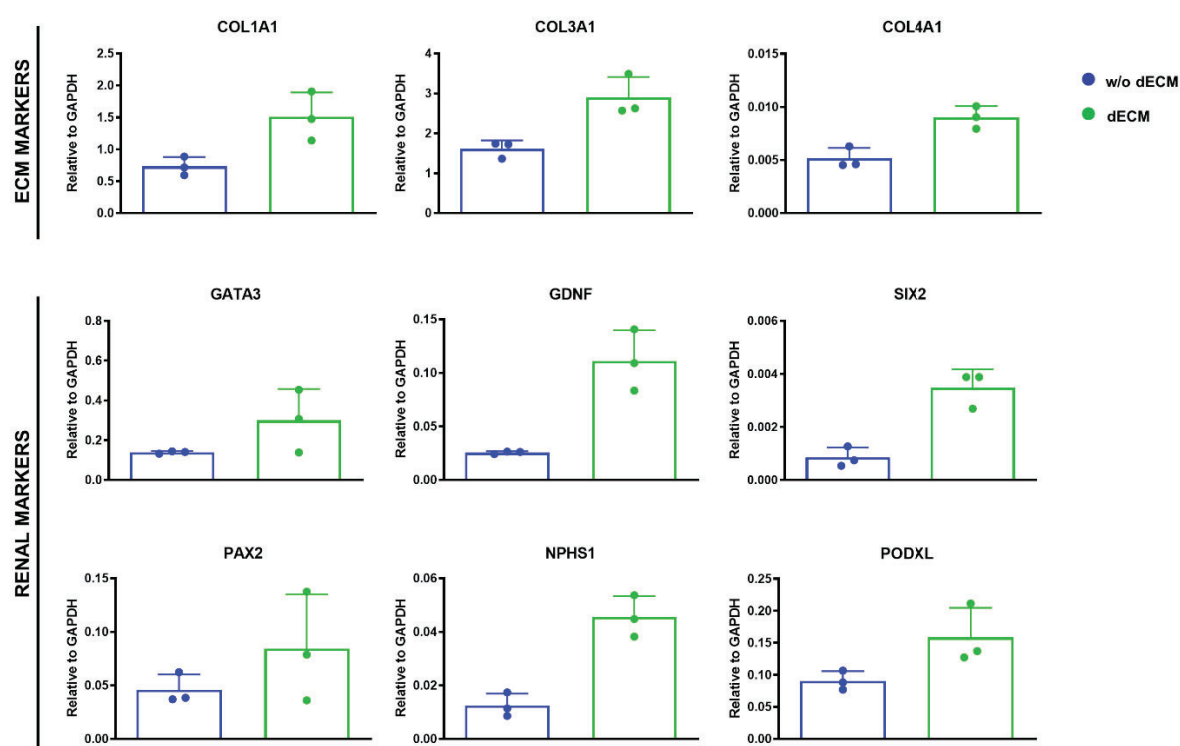


Fig. 39. Characterization of encapsulated hPSCs-kidney progenitor cells within renal dECM-derived hydrogels through qPCR analysis. Representative images of qPCR analysis for the indicated makers in *D20* hPSC-kidney organoids derived from *D4* hPSCs-IM-like cells encapsulated without (blue) and with (green) renal dECM-derived hydrogels. Data are mean $\pm$ SD. $n = 1$ independent biological replicate with 3 technical replicates each.

4.5.5. Generation of hPSCs-derived CLCs exploiting cardiac dECM-derived hydrogels in 2D cell culture conditions

Cardiac tissue engineering aims to generate biomaterials preserving cardiovascular properties, including elasticity, conductivity, and contractility of cardiomyocytes while seeking to enhance the survival and retention of cardiomyocytes and stem cells *in situ*. Due to their biocompatibility and biomimetic nature, hydrogel-based biomaterials have gained attention for cardiac regeneration. Based on these previous observations, we aimed to explore the potential use of cardiac dECM-derived hydrogels on the generation of hPSC-derived CLSCs. Briefly, beating *D12* hPSCs-derived CLCs were grown without and with cardiac dECM-derived hydrogels diluted in the cardiac cell culture media from *D12* until *D35* (Fig. 40A). At *D35* mRNA expression levels of the cardiac markers *MYL2*, *SIRPA*, *TNNT2* and *TNNI3* were increased when cardiac dECM-derived hydrogels were used in front of 2D control conditions (without cardiac dECM-derived hydrogel) (Fig. 40B). Instead, cardiac ion channel markers did not show significant differences between the two conditions.

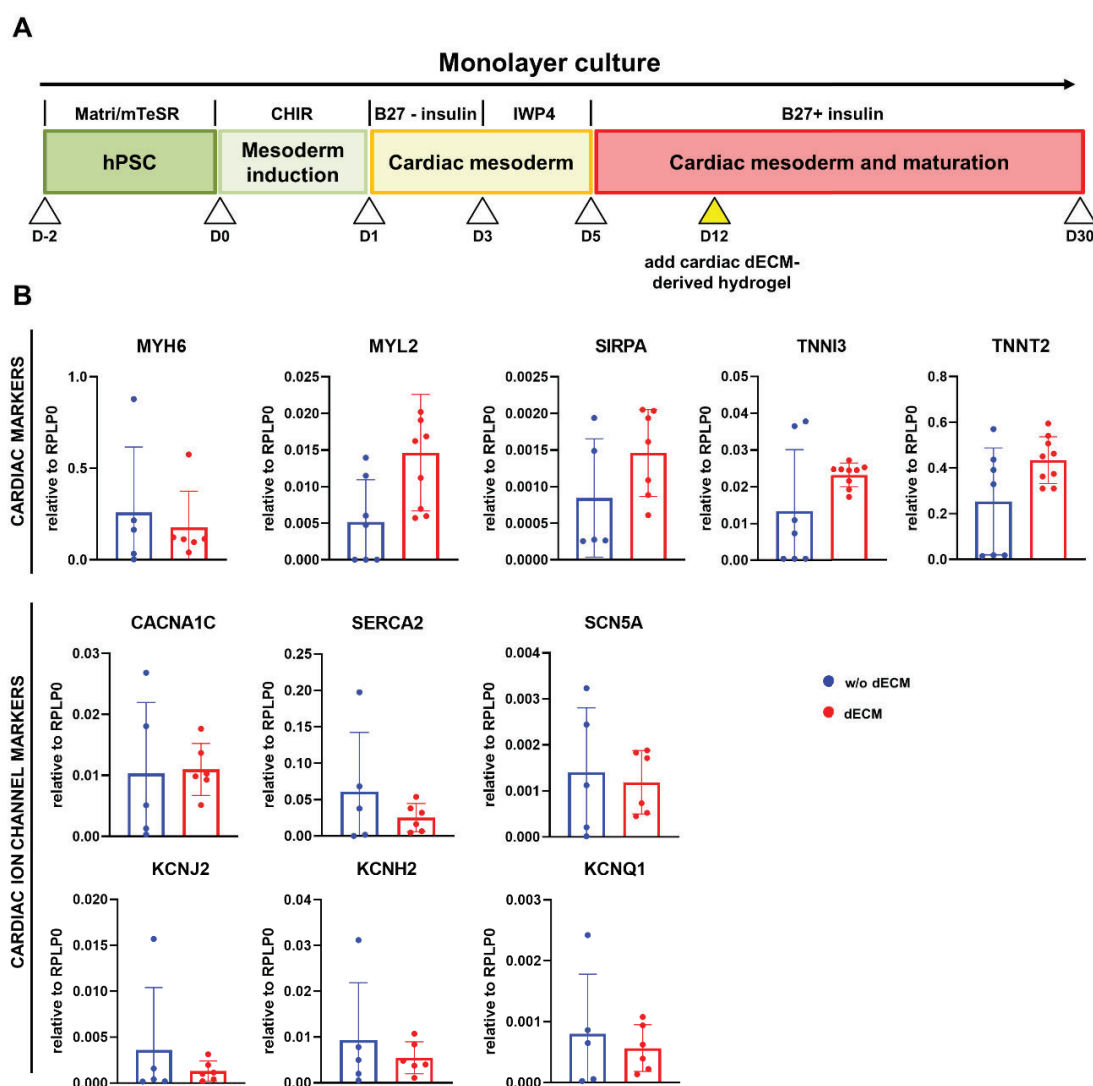


Fig. 40. Characterization of hPSCs-derived CLCs exploiting cardiac dECM-derived hydrogels in 2D cell culture conditions by qPCR analysis. **A)** Experimental scheme for the generation of hPSC-derived CLCs kept in culture in the absence (blue) or presence (red) of cardiac dECM-derived hydrogel in the cell culture media from *D12* until *D35*. **B)** Representative images of qPCR analysis for the indicated cardiac and cardiac ion channel makers in *D35* hPSCs-derived CLCs supplemented without (blue) and with (red) heart dECM-derived hydrogels. Data are mean $\pm$ SD. *n* = 2 independent biological experiments with 3 technical replicates each.

4.5.6. Generation of hPSCs-derived cardiomyocytes exploiting cardiac dECM-derived hydrogels in 3D floating condition

To further explore on the potential impact of cardiac dECM-derived hydrogels on the extent of hPSCs differentiation into the cardiac lineage in the 3D setting, *D12* beating cardiomyocytes were detached in single cells and further aggregated by spin centrifugation to form spheres of approximately 50.000 (50K) cells. From *D13*, 50K cardioids were grown without and with cardiac dECM-derived hydrogels diluted in the

media (Fig. 41A). Deeper analysis were performed through qPCR highlighting higher expression not only of cardiac markers (*MYH6*, *SIRPA*, *TNNT2* and *TNNI3*), but also an up-regulation of specific cardiac ion channel markers, such as *CACNA1C*, *KCNJ2*, *SERCA2*, *SCN5A*, *KCNH2* and *KCNQ1*, suggesting the helpful role played by the heart dECM during cardiac differentiation (Fig. 41B).

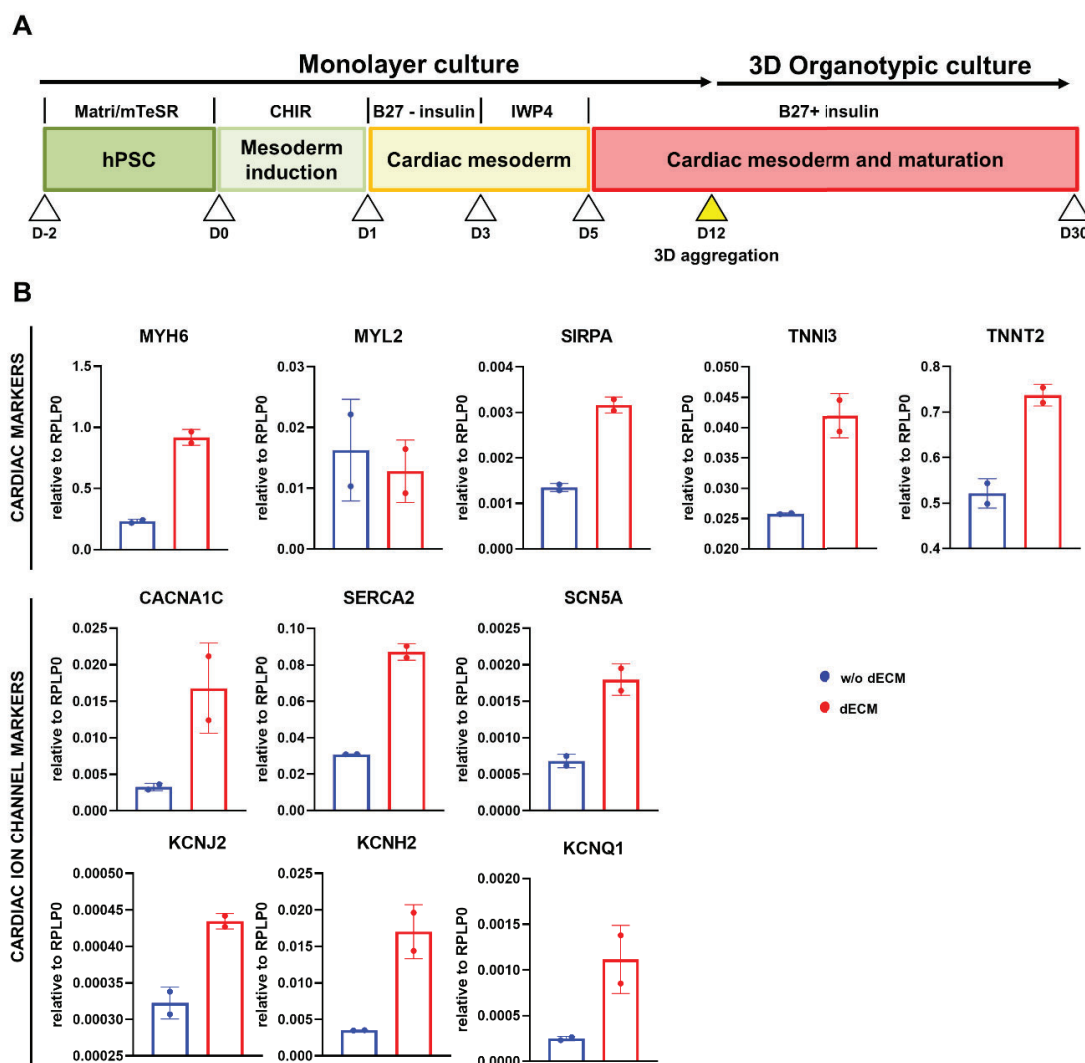


Fig. 41. Characterization of 50K floating organoids with cardiac dECM-derived hydrogel by qPCR. A) Experimental scheme for the generation of cardiac-like organoid constructs in 3D in the absence (blue) or presence (red) of cardiac dECM-derived hydrogels. First, cardiac mesoderm was induced in hPSCs by activation and inhibition of Wnt pathway (CHIR and IWP4). After enzymatic dissociation *D12* beating monolayers were aggregated in 3D as cardiospheres of 50K and kept in culture after *D35*. **B)** Representative images of qPCR analysis for the indicated cardiac and cardiac ion channel makers in *D35* 50K floating organoids supplemented without (blue) and with (red) cardiac dECM-derived hydrogels. Data are mean $\pm$ SD. *n* = 1 independent biological replicate from a pool of 12 organoids/group with 2 technical replicates each.

Further culture conditions were explored to assess the impact of cardiac dECM-derived hydrogels in the differentiation of hPSCs into cardiac-like cells in the 3D setting. To that *D12* beating cardiomyocytes were detached in single cells and further aggregated by spin centrifugation to form cardiospheres of approximately 5000 (5K) cells in aggrewell. After 24 h, 5K cardiospheres were seeded on a thick layer of collagen without and with cardiac dECM-derived hydrogels using the trans-well culture system (insert). The resulting cardiac-like constructs were cultured until *D35* in the absence or presence of cardiac dECM-derived hydrogel in the cell culture media which exhibit synchronic beating from *D15* (Fig. 42A). qPCR analysis highlighted higher expression of cardiac markers (*MYH6*, *SIRPA*, *TNNT2* and *TNNI3*), but such as the 2D cell culture condition, a similar expression of cardiac ion channel markers in the two conditions (Fig. 42B).

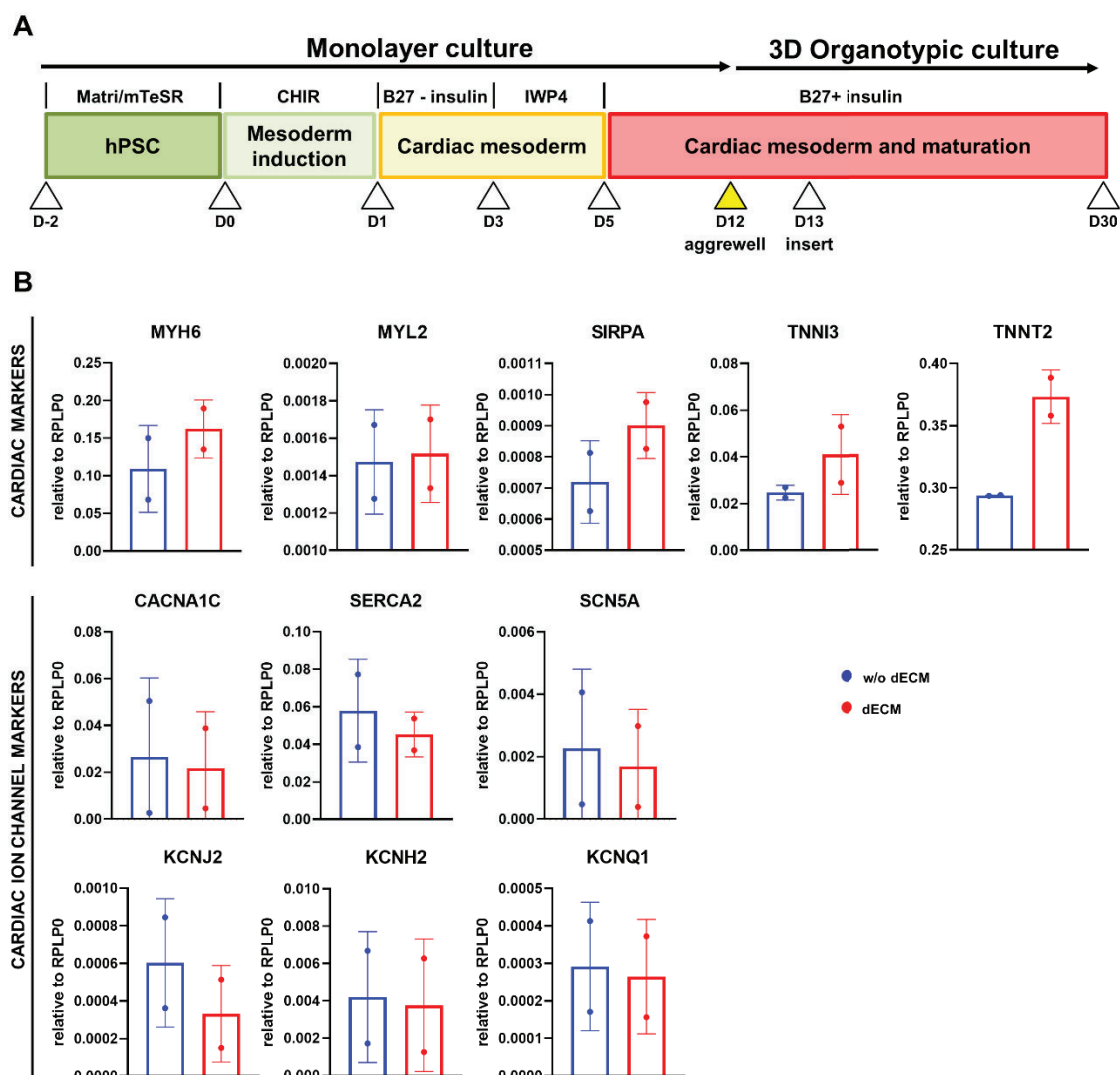


Fig. 42. Characterization of cardiac differentiation in insert with cardiac dECM-derived hydrogel by qPCR. A) Experimental scheme for the cardiac differentiation in 3D-constructs in insert: cardiac mesoderm was induced by activation and inhibition of Wnt pathway (CHIR and IWP4) such as in 2D conditions. 5K self-assembled 3D organoids were aggregated at *D12* in aggrewell and on *D13* seeded on a thick layer of collagen mixed with/without cardiac dECM-derived hydrogel maintained until *D35*. **B)** Representative images of qPCR analysis for the indicated cardiac and cardiac ion channel makers in *D35* cardioids in insert supplemented without (blue) and with (red) cardiac dECM-derived hydrogels. Data are mean $\pm$ SD. $n = 2$ independent biological experiments from a pool of 12 organoids/group with mean of 3 technical replicates each.

DISCUSSION

5. DISCUSSION

Human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) (1) and human induced pluripotent stem cells (hiPSCs) (2), have emerged as a promising and alternative platform to animal models in recent decades for the study of tissue development and function, as well as to model human disease. Significantly, their inherent capacity to self-organize into tissue-like structures resembling native organs has opened new avenues of research. This breakthrough has led to the development of hPSC-derived organoids, three-dimensional models that accurately recapitulate relevant features of human development, physiology, and disease, offering a more realistic representation of human biology. When combined with genetic engineering techniques, these cell model systems provide an unprecedented opportunity to unravel the underlying genes and molecular mechanisms behind complex human traits. Notably, the development of genetic engineering techniques, such as the CRISPR/Cas9 system, has revolutionized the precise and straightforward manipulation of the hPSC genome (192). This convergence of hPSC technology and genetic engineering presents new avenues for understanding human diseases at the molecular level.

By leveraging the potential of hPSCs and genetic engineering, here we have focused our attention on the generation of reporter cell lines for cardiac genes exploiting our recently developed iC2 platform which allows for genetic engineering in hPSCs, including the generation of stable and inducible knock-out, knock-in and reporter cell lines. This approach has allowed to first establish reporter cell lines targeting *MYH6*, *MYL2* and *SIRPA* loci and further dissect and exploit MYH6 hPSCs reporter cell lines to set up new procedures of cardiac differentiation and cardiac disease modelling. In this line, other studies already pointed the amenability of this approach to exploit both murine and human hESCs. For the introduction of reporter markers (either antibiotic resistance genes or fluorescent reporters), the first approaches utilized integrative methods. In this regard, early in 2009 Kita-Matsuo et al. already demonstrated the feasibility of lentiviral vectors for expressing and selecting cardiac reporter genes in hESCs (211). In that study, the authors generated hESCs reporter lines for both Brachyury (*T*) and *MYH6* promoter sequences with mCherry or GFP fluorescent

proteins and antibiotics resistance under the promoter of *Rex1*. In this way, it could be possible a more precise tracking and monitoring of cardiac differentiation process by following two different cell populations. Using a similar approach, α -MHC-GFP hESC reporter line was generated through lentiviral vectors in 2011 by Ritner et al. (268). Clearly, α -myosin heavy chain (α -MHC or MYH6), expressed in early and late stages of cardiac differentiation, has been used many times as targeted focus to generate transgenic mice or edited cell line using α -MHC promoter to drive a reporter expression. In this regard, making use of TALEN and CRISPR advances, the host laboratory decided to target MYH6 *locus* and generate a MYH6-mCherry/w hESC line in order to define a robust cardiac differentiation *in vitro* protocol (56). Moreover, to study the segregation of cardiac progenitors many studies focus gene targeting on MESP1 transcription factor. This is a novel marker of early cardiac progenitors and many studies have used it to create human cardiac reporter lines. Targeting MESP1 genomic *locus* by HR with a construct containing mCherry reporter gene provides a great source to define MESP1-mCherry hESC lines. The analysis of these MESP1-mCherry⁺ cells showed gene expression pattern of mesodermal character (269). Another reporter cell line created by Elliott et al. in 2011 provided great advances in cardiac differentiation study. NKX2.5-eGFP/w hESCs line consist of eGFP reporter gene integration into NKX2.5 *locus* enabling cardiac progenitor and CM purification (233). Moreover, the generation of dual reporter cell lines like MESP1 mCherry/w-NKX2-5 eGFP/w hESCs has shown to facilitate the understanding of early specification from cardiac lineages to CM fate commitment signalling pathways (207). Successively, since the emergence of CRISPR/Cas9 technology, several cardiac reporter cell lines were generated. Sharma et al. developed TTN-GFP reporter cell lines for the study of sarcomere function (270) or more recently, an mCherry reporter was introduced under the MYH6 *locus* of H1 and H9 hESC lines by applying CRISPR/Cas9 technology as monitor for drug-induced cardiotoxicity (271). Nevertheless, CRISPR/Cas9 technology started to encounter certain limitations, such as plasmid-based editing approaches. In order to address these limitations, researchers have made efforts to enhance and refine this approach. In 2014, Gonzalez et al. developed the iCRISPR platform (199). This innovative platform harnesses the inherent capability of the human AAVS1 *locus* to facilitate reliable and long-lasting transgene expression. The methodology involves targeting both alleles of the AAVS1 *locus* using TALEN-mediated knock-in with the aid of two donor vectors.

Leveraging the iC1 platform, the host laboratory has made significant advancements by introducing an enhanced version known as iC2 (229). This upgraded platform offers numerous advantages over conventional gene editing tools for hPSCs. Firstly, the occupancy of only a single AAVS1 *locus*, which streamline the editing process. Secondly, the flexibility to induce gene editing at any desired time through the induced activation of Cas9 through DOX treatment. Lastly, the iC2 platform boasts exceptional efficiency, ensuring high success rates in the editing procedure. The reporter cell lines described in this thesis take advantages of the improvement of the iC2 platform. We have successfully generated hPSC reporter cell lines for 3 distinct cardiac genes expressing at different time during cardiac development, namely *MYH6*, *SIRPA* and *MYL2*. The use of these hPSCs provides several advantages. Firstly, they enable monitoring the endogenous expression of this markers throughout all stages of cardiac differentiation. Additionally, the ability to isolate positive cells for the different markers at different stages of cardiac differentiation using techniques such as FACS. In this manner it would be possible to further open new possibilities to molecularly dissect the transcriptomic landscapes and gene regulatory networks necessary for cardiac lineage initiation (*MYH6*) and cardiomyocyte differentiation (*MYH6*, *SIRPA* and *MYL2*). Importantly, this approach has been extensively tested and confirmed in both male and female hPSCs, including hESCs and hiPSC lines.

To evaluate the feasibility of the reporter cell lines generated here to recapitulate cardiac differentiation, undifferentiated hPSCs cardiac reporter lines were exposed to cell culture conditions that firstly induce and subsequently inhibit the Wnt signaling pathway to initiate commitment towards mesoderm fate, following a well-established cardiac-like differentiation protocol developed previously in the host laboratory (56). Similarly, other laboratories took advantage of similar strategies for the derivation of cardiac-like cells showing spontaneous beating in 2D cell culture conditions. For instance, one of the first protocols for the derivation of hPSCs-derived cardiac-like cells established a co-culture system in which hESC were cultured with visceral endoderm-like cells which resulted in low cardiomyocyte-like cells yield and purity (116). Successively, and thanks to the advances on cardiac developmental biology from studies in the mice model, new studies took advantage of growth factors to further mirror biochemical-related signalling known to specify and instruct cardiac differentiation (i.e., activin A, FGF2, and BMP4) (113). Those early protocols, which

demonstrated superior efficiency in differentiating cardiomyocytes compared to spontaneous conditions, marked an early application of one of the most widely used cardiac basal media combination in current times (RPMI/B27). The successful utilization of this media set a precedent and established it as an almost standard choice in subsequent years. Additionally, the first chemically-defined medium for cardiomyocyte differentiation was developed, revealing the potential negative impact of insulin for mesoderm induction (272). Consequently, in current protocols including ours, insulin is not used during the first stages of the cardiac differentiation process (56). Further advancements focused on enhancing differentiation through strategies such as Wnt inhibition (273), optimizing growth factor concentrations combined with fetal bovine serum (274), identifying stage-specific signaling requirements during development (114), and refining chemically-defined conditions. Taking into account all these findings, the host laboratory developed a protocol that utilizes a chemical inhibitor of GSK3 β to activate endogenous Wnt signaling, while suppressing the expression of pluripotent-related genes after 3 days. It is worth noting that inhibiting the Wnt pathway during this stage can potentially trigger the activation of cardiac progenitor genes. Overall, by refining various parameters, such as the number of undifferentiated cells plated and the dimension of the cell colonies, a successful cardiac differentiation protocol has been established. The differentiation of our hPSCs cardiac reporter cell lines using this protocol allowed the appearance of MYH6 positive cells as early as *D7* after initiating differentiation, followed by the expression of SIRPA at *D21* and MYL2 around *D30* of the differentiation process. The development could be monitored in real-time based on the detection of the fluorescent proteins linked to the expression of the cardiac reporter genes, providing valuable insights into the progressive stages of differentiation. Moreover, we demonstrated that undifferentiated cardiac reporter cell lines preserved the different morphological and phenotypic properties associated with hPSCs biology, such as pluripotent-related markers expression or capacity to give rise to cells from the three germ layers, among others. Building upon our previous findings regarding the utilization of cardiac human pluripotent stem cell lines for monitoring cardiac differentiation in 2D cell culture, another objective was to establish a robust and scalable protocol for generating self-assembling cardiac-like cells within 3D culture environments. Our approach has allowed to develop a high-throughput, reproducible, and straightforward approach for generating 3D cardiac-like cultures based on morphological, transcriptomics and

functional characterization. In the last few years, there has been a notable increase in the development of cardiac organoid protocols. In accordance with the objectives of this thesis, various approaches have been developed to generate intricate multilineage organoids that consist of structures expressing both mesoderm and endoderm lineages (129). Furthermore, procedures have been established to generate hPSCs-cardiac organoids that exhibit cavities or chamber-like structures resembling the embryonic heart (41,128). More recently, attention has been also directed towards the generation of protocols specifically focused on generating enhanced and well organized epicardial structures within hPSCs-cardiac organoids (134). Additionally, Aguirre laboratory, designed another approach for the development of hPSCs-cardiac organoids with higher anatomical complexity and physiological relevance, closely resembling the developmental steps observed during the first trimester of fetal development *in utero* (275). Building upon previous findings, nearly all procedures, including the one employed in that study, utilize the chemically-defined medium for cardiomyocyte-like cells generation in 3D culture conditions. Initially, the medium is supplemented without insulin, due to previous findings confirming the inhibiting role of insulin during the first stages of cardiomyocyte differentiation (272,276). From *D5* on, it is supplemented with insulin, alongside specific factors that induce activation or inhibition of the Wnt signalling pathway. On the other hand, the self-assembling process for generating cardiac organoids takes place at different time points in each protocol. Some protocols allow the aggregation of hPSCs-derived cardiac cells during the early stage of mesodermal differentiation as soon as during *D-2* and *D-1* (41,126), while others at later stages, when the monolayer has already started beating, such as *D5* and *D15* (127,129). In our study, we decided to aggregate cardiac organoids on *D3*, coinciding with the induction of Wnt pathway inhibition, trying to allow the self-assembly only for the cells that have been going towards cardiac mesoderm.

In this thesis we provide a simple and highly reproducible protocol to generate physiologically relevant cardiac-like organoids with high structural and multicellular complexity using hPSCs. Moreover, our cardiac-like organoids were effectively derived from both hiPSC and hESC lines, showing their reproducibility. These heart-resembling organoids exhibit the ability to self-organize in 3D environment at *D3* of cardiac differentiation. Importantly, our organoids initiated functional beating by *D10* and can be maintained up to *D100* in cell culture with a long-term functional beating in

absence of external stimuli. The efficacy of our protocol was initially validated through comprehensive expression analysis, encompassing not only cardiac but also ion channel, endothelial, and metabolic markers within our cardiac-like organoids. Immunofluorescence analysis revealed the presence of the predominant gap junction channel-forming protein in cardiomyocytes, Connexin 43 and the characteristic z-lines observed in sarcomere structures, marked by α -Sarcomeric Actinin. It has already been demonstrated that sarcomeres tend to be longer in mature cardiomyocytes (246). Indeed, we noted a noticeable increase in sarcomeric length within *D60* cardiac-like organoids in comparison to their *D30* counterpart. Notably, these sarcomeric lengths closely aligned with previously reported findings falling within the expected range (127,248).

Moreover, within our cardiac-like organoids we observed endothelial markers at both mRNA and protein levels without requiring any supplementary interventions in the protocol. This achievement holds substantial importance, given that the absence of a functional vascular network remains a major constraint in numerous organoid systems.

In addition, size measurement analysis unveiled a gradual reduction in organoid size throughout the differentiation process, accompanied by a concurrent accumulation of collagen fibers. These findings suggest a progressive development of ECM contributing to the establishment of a robust tissue structure and support. Ultrastructural images of our organoids revealed a morphology characterized by the distinctive organization and maturation commonly associated with cardiac muscle sarcomeres. Calcium transient analysis performed at various time points corroborated the maturation of our cardiac-like organoids throughout the differentiation process. The observed heightened frequency coupling at *D30* and *D60* strongly implies that these organoids, in their later stages, exhibit functional properties closely resembling those of authentic cardiomyocytes.

Although qPCR and immunofluorescence data indicate the presence of different cell types, these techniques might not comprehensively capture the heterogeneity of our cardiac-like organoids. In this regard, taking advantages of the advances in single-cell and single-organoid “omics” technologies of the recent years, a single-cell transcriptomic analysis of cardiac-like organoids at early (*D30*) and late stage (*D100*) provided us with valuable insight into the dynamic changes within cell population. At

early stage, cardiac-like organoids exhibited characteristics of cardiomyocytes that further develop towards more specific atrial, ventricular and fibroblast cell-specificity expression at late stage. As the development progressed towards the late stage, we observed a remarkable transformation, with the cells displaying more specific expression profiles for atrial, ventricular, and fibroblast cell types. This observation suggests a maturation process where the organoids acquire distinct cell-specificity expressions, reflecting the sophisticated organization and differentiation within the cardiac-like structures. Overall, we propose the establishment of a robust and reproducible cardiac-like organoid differentiation protocol that recapitulate the cellular complexity and maturation process of the native human heart.

Exploiting the newly defined conditions for the development of cardiac-like organoids, we focused our attention on the potential use of this system to model early hallmarks of diabetic cardiopathy. Diabetes mellitus is one of the most common multi-systemic diseases and it is characterized by high blood sugar levels due to insufficient insulin production or impaired insulin function (277). In recent decades, the prevalence of diabetes has experienced a remarkable surge, affecting nearly 500 million people worldwide. This alarming increase has had profound implications, with cardiovascular complications associated with diabetes leading to approximately 5 million deaths. Diabetic cardiomyopathy, the most frequent cardiovascular complication of diabetes, presents with initial signs of diastolic dysfunction and left ventricular hypertrophy, followed by the progression to systolic dysfunction and eventual development of heart failure (225). The establishment of an *in vitro* model that faithfully replicates crucial aspects of diabetic cardiomyopathy would serve as a valuable tool for studying underlying disease mechanisms, enhancing our comprehension of the condition, and potentially advancing treatment strategies for patients. hPSC-derived cardiac-like organoids hold great promise for developing an effective model to unravel the intricate complexities of diabetic cardiomyopathy. Currently, there are not many findings related to the use of cardiac-like organoids to model diabetes, except for a study by Lewis-Israeli et al. In their work, the authors successfully modelled the impact of pre-gestational diabetes by subjecting the so-called heart organoids aggregated at *D-2* to different levels of glucose and insulin (3.5 mM and 170 pM insulin for the normoglycemic and 11.1 mM glucose and 1.14 nM insulin for the diabetic-like condition) from *D0* to *D6* of cardiac differentiation (41). In this thesis, we subjected

cardiac-like organoids to cell culture conditions inducing early hallmarks of diabetes as recently published by the host laboratory (227). Our model is based in the exposure of organoids to normoglycemia (5 mM glucose) or oscillatory glucose levels (5- 25 mM glucose) for one week. We have tried different moments (*D21* and *D60*) during the onset of differentiation to perform these interventions. Several outputs have been measured to further assess the extent of phenotypic changes associated with early hallmarks of diabetic cardiopathy, including alterations in protein expression and ECM remodeling by immunohistochemistry and confocal microscopy analysis with image quantification, transcriptional analysis by qPCR, and Seahorse analysis. First, our results showed that metabolic insults did not alter cardiac marker expression during the cardiac differentiation process, underscoring the robustness of our cardiac-like organoids as human disease model for early hallmarks of diabetes. Moreover, also if at mRNA levels we could not see significant differences, our model showed that at protein level, diabetic *milieu* induces ECM alterations, such as collagen fiber accumulation in diabetic-like organoids compared to normoglycemic condition. We could observe also a metabolic shift associated with a decreased oxygen consumption rate (OCR) in diabetic-like cardiac-like organoids and enhanced metabolic activity associated with increased metabolic signatures related to fatty acid oxidation (FAO), mitochondrial respiration, and glycolysis in normoglycemia. Data also confirmed at subcellular levels by TEM showing the higher number of lipid droplets in diabetic-like condition. At functional level, our findings suggest that a diabetic-like *milieu* disrupt calcium homeostasis in cardiac-like organoids, potentially compromising cardiac contractility and predisposing the myocardium to dysfunction. In summary, the normoglycemic condition displayed better coupling and functional characteristics compared to the diabetic-like one. This investigation highlights the arrhythmic events and abnormal contraction typical of patient affected by diabetic cardiomyopathy. Overall, our findings reveal significant metabolic, mitochondrial, molecular, and ECM alterations between normoglycemic and diabetic-like samples, characteristic early indicators of diabetic-related diseases. These discoveries validate the use of our cardiac-like organoids as human disease model for diabetic-related conditions.

The last part of this thesis exploited the use of biomaterials to improve the extent of organoid differentiation. Recently, there has been growing interest in studying the extracellular matrix due to its functional properties, specifically its ability to generate

signals that influence cell fate and differentiation (42). In particular, the use of decellularized ECM (dECM) that aims to mimic the native microenvironment of a tissue or organ could offer a promising solution. In the renal scenario, recent findings from Kim et al. showed the development of more mature glomeruli by co-culturing kidney dECM hydrogel with hPSC-derived kidney organoids analyzed by single-cell RNAseq profiling (52). This approach combined the kidney organoid differentiation protocol developed by the Freedman laboratory (173) with the application of a kidney-derived dECM hydrogel and supplemented the media with VEGF factor, to enhance the vascular network. Additionally, Kim et al. introduced the use of kidney dECM from the early stages of the differentiation process. We obtained similar results when utilizing a human kidney dECM-derived hydrogel generating through immersion agitation decellularization process. In our case, the hydrogel was combined with biologically inert materials, such as fibrinogen and gelatin, facilitating the growth of hPSC-kidney derived organoid constructs but not affecting the kidney differentiation process. Our approach consisted into the mixing of the hydrogel with the *D4* IM-like cells derived from a kidney differentiation protocol established in the host laboratory (39). Notably, the construct with dECM showed higher expression of renal markers, indicating an elevated level of maturation. Furthermore, even without supplementing the media with factors that enhance angiogenesis, our kidney organoids cultured with dECM-derived hydrogel exhibited a more developed endothelial program compared to the control condition.

On the other hand, in cardiac regenerative medicine field, the advancements in bioengineering are making the building of an engineering bio-artificial heart increasingly feasible, thereby addressing the critical shortage of donor organs for transplantation. Since the achievement of decellularizing whole rat heart in 2008, acellular scaffolds have emerged as an optimal tool for tissue engineering (278). Ten years later, the studies carried out by Taylor and Noor have set the basis to achieve this important milestone. In the work of Taylor et al., they conducted experiments involving the implantation of porcine decellularized hearts in live recipients in a position different from their natural location. This work aimed to investigate the impact of the surgical procedure on preventing coagulation and enhancing the viability of the graft. But more interestingly, the study exploited the recipient's inherent ability to repair and regenerate tissue by utilizing the acellular scaffold. This scaffold acted as a framework,

providing essential cues that guided the organization and functioning of host cells within the transplanted organ. This approach allowed the transplanted scaffold to direct the recipient's cells, promoting their organization and remodelling within the transplanted organ. Overall, the study highlighted the potential of utilizing decellularized organ scaffolds to harness the recipient's regenerative capabilities and facilitate successful organ transplantation (279). On the same line, Noor et al. used decellularized omental tissue to generate personalized materials as bioink for 3D printing. The technology demonstrates the potential to engineer vascularized cardiac patches that perfectly match the immunological, cellular, and biochemical properties of the patient. Importantly, since the bioinks come from the same individual, there is no risk of immune rejection after transplantation, eliminating the need for immunosuppression treatment. Additionally, a customized formulation enables the printing of complex biological structures with the personalized hydrogel. This approach paves the way for engineering acellularized hearts with a natural architecture, offering potential for organ replacement and drug screening in a physiologically relevant structure (280). In 2016, the host laboratory achieved a significant breakthrough by utilizing perfusion decellularization techniques to remove cellular components from human hearts, allowing for the co-culture of cardiomyocytes derived from hPSCs cells (56). Previously, another study demonstrated that the encapsulation of differentiated hESC-derived embryoid bodies (EBs) with ECM hydrogel led to increased expression of cardiac markers (55). Building upon these important findings, we employed a similar experimental set-up to that defined for the generation of hPSC-kidney derived organoid constructs. Our aim was to establish new culture conditions that would enhance the generation of 2D and 3D cardiac-like cell cultures. To that we made use of immersion and agitation decellularization process for the generation of decellularized scaffolds from porcine hearts that meet the established criteria of an optimal tissue decellularization. Our method successfully removes cellular material while preserving the structural integrity and composition of the ECM. These data were confirmed quantitatively by DNA quantification, in which we obtained minimal residual of DNA at the end of the decellularization protocol confirming the success of our process. Furthermore, our investigation utilized histological and immunocytochemistry analysis, which provided compelling evidence for the well-preserved tissue structure represented by the ECM. Notably, the absence of cellular components, such as nuclei, further attests to the thoroughness of our decellularization approach. To assess the

impact of cardiac dECM-derived hydrogels on cardiac differentiation and maturation, we made use of this hydrogels to enrich our cell culture media from *D12-13* in both monolayer and cardiac-like organoids cell culture conditions. To provide further insights with regards to this important issue, we generated also cardiosphere-like constructs seeded on a collagen layer combined with cardiac dECM-derived hydrogel. In both 2D and 3D cell culture conditions, we obtained higher expression of cardiac markers at the end of cardiac differentiation process in the condition with cardiac dECM-derived hydrogel. Also in this case, our results showed the beneficial impact played by the heart dECM on the differentiation process.

Despite the exciting opportunities our technology offers for *in vitro* modelling of human heart diseases and improving kidney and heart differentiation protocols, there are still some limitations. Firstly, while our cardiac-like organoids hold the advantage of being derived from human cells rather than surrogate animal models like mice, their ability to fully replicate specific heart development remains limited compared to other existing cardiac models in which the formation of specific structures has been demonstrated, such as chamber-like structures (41,128). Secondly, the extended duration of each experiment (up to 100 days) has restricted the time for a comprehensive characterization and analysis of additional features, such as the examination of epicardial structures (134). Undoubtedly, there is still a long path ahead.

For instance, as previously mentioned, a critical hurdle for organoid models is the possibility to generate an organized endogenous vascularization. Although we have detected endothelial markers within our cardiac-like organoids, further studies and enhancement focusing on the functionality of these vascular tissues would be required. Specifically, there is a need to ascertain the maturity of the vessels and their degree of connectivity. One promising future perspective for the achievement of these aims is through the fusion with endothelial-derived organoids (assembloids). In this line, current efforts in the host laboratory have already achieved significant progress in the generation of kidney assembloids by the manual assembly of hPSC-endothelial organoids with hPSCs-kidney organoids demonstrating a clear enhancement in the self-organization within glomerular- and tubular- like structures. This was obtained by assembling both organoid models in organotypic culture conditions and in free floating culture conditions (*Garreta et al., unpublished*). Other approaches to further enhance the organization of endogenous vascularization can be addressed through techniques

such as implanting organoids onto the chorioallantoic membrane (CAM) or employing organ-on-chip platforms. Because its easy accessibility and well-defined vascularization, CAM model is one of the widely used *in vivo* approaches for the study of angiogenesis (281). In this regard, the host laboratory developed a transplantation method in CAM providing vascular component for kidney organoids (39).

Finally, one of the notable limitations concerning the new procedures to derive organ-derived hydrogels from decellularized extracellular matrices from heart and kidney, is the inconsistency observed among different batches which could hindered the proper differentiation process. While the use of dECM has shown promising results in promoting cellular behaviour and tissue regeneration, the lack of uniformity across batches poses challenges in ensuring reproducibility and reliability in experiments.

Nevertheless, despite these challenges and limitations, when we take a picture of all the collective findings, we could describe the generation and characterization of cardiac reporter cell lines using CRISPR/Cas9 technology, allowing us to monitor the activity of specific cardiac transcriptional factors during the process of cardiac differentiation. Building upon this achievement, we examined the suitability of these reporter cell lines for further differentiation experiments. Initially, we utilized an already established two-dimensional cardiac differentiation protocol from the host laboratory. Subsequently, we developed a novel three-dimensional differentiation protocol to generate cardiac-like organoids mimicking the cellular complexity and maturation process of the native human heart. Expanding the potential of our cell culture platform, we sought to explore its utility in modelling early hallmarks of diabetic heart disease. To achieve this objective, we subjected the cardiac organoids to specific cell culture conditions that induce a diabetic-like phenotype, previously established for kidney organoids. Here, we further expand the use of these treatments at different stages of cardiac-like organoids culture also promoting different extents of diabetic-like responses. Finally, we harnessed on the derivation of hydrogels from both native heart and kidney tissues. Through an immersion and agitation tissue decellularization process, we successfully defined new procedures for the derivation of organ-derived hydrogels from decellularized extracellular matrices. By combining these innovative biomaterials with hPSC-derived organoids, we established novel model systems with immense potential for enhancing the differentiation process through the development of improved culture conditions. In conclusion, this thesis proposed cutting-edge hPSC-

derived biomodels, which serve as valuable resources for conducting promising research and paving the way for future advancements in regenerative medicine applications.

CONCLUSIONS

6. CONCLUSIONS

1. We have successfully generated MYH6-GFP hESC and MYH6-mOrange, MYL2-mOrange, SIRPA-mOrange hiPSC reporter lines by exploiting a recently developed iC2 platform.
2. We have validated the functionality of hPSC cardiac reporter lines by employing a well-established 2D cardiomyocyte-like differentiation protocol. The use of the hPSC cardiac reporter lines enabled the monitoring of endogenous expression of MYH6, MYL2 and SIRPA markers throughout all stages of cardiac differentiation.
3. We have defined a novel approach to generate 3D cardiac-like organoids in a robust and efficient manner exhibiting relevant cardiac differentiation and functional characteristics.
4. We have established a new cardiac *in vitro* model that can emulate important aspects of diabetic cardiomyopathy taking advantages of the newly defined cardiac-like organoids. Cardiac-like organoids exposed to diabetic-like culture conditions compared to normoglycemia analyzed at morphological, transcriptional, and functional levels, showed potential phenotypic alterations associated to early hallmarks of diabetic cardiopathy.
5. We have defined novel procedures based on organ decellularization technology to fabricate biomaterials mimicking the biochemical composition of the native organ. Our findings showed the successful derivation of biomimetic hydrogels from decellularized extracellular matrices (dECM) obtained from kidney and heart tissues.
6. We have established new conditions for the generation of complex kidney organoid systems based on the use of renal dECM-derived hydrogels. Our results showed higher level of maturation in kidney organoid constructs in presence of renal dECM-derived hydrogels.
7. We have established new conditions for the generation of 2D and 3D cardiac-like cell cultures based on the use of cardiac dECM-derived hydrogels. Our results showed higher level of maturation in cardiac-like cells in presence of cardiac dECM-derived hydrogels.

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ANNEXES

8. ANNEXES

Co-authored scientific publications

- Selfa IL, Gallo M, Montserrat N, Garreta E. Directed Differentiation of Human Pluripotent Stem Cells for the Generation of High-Order Kidney Organoids. *Methods Mol Biol.* 2021;2258:171-192. doi: 10.1007/978-1-0716-1174-6_12. PMID: 33340361.
- Garreta E, Prado P, Stanifer ML, Monteil V, Marco A, Ullate-Agote A, Moya-Rull D, Vilas-Zornoza A, Tarantino C, Romero JP, Jonsson G, Oria R, Leopoldi A, Hagelkruys A, Gallo M, González F, Domingo-Pedrol P, Gavalda A, Del Pozo CH, Hasan Ali O, Ventura-Aguilar P, Campistol JM, Prosper F, Mirazimi A, Boulant S, Penninger JM, Montserrat N. A diabetic milieu increases ACE2 expression and cellular susceptibility to SARS-CoV-2 infections in human kidney organoids and patient cells. *Cell Metab.* 2022 Jun 7;34(6):857-873.e9. doi: 10.1016/j.cmet.2022.04.009. Epub 2022 May 12. PMID: 35561674; PMCID: PMC9097013.
- Garreta E, Moya-Rull D, Stanifer ML, Monteil V, Prado P, Marco A, Tarantino C, Gallo M, Jonsson G, Hagelkruys A, Mirazimi A, Boulant S, Penninger JM, Montserrat N. Protocol for SARS-CoV-2 infection of kidney organoids derived from human pluripotent stem cells. *STAR Protoc.* 2022 Dec 16;3(4):101872. doi: 10.1016/j.xpro.2022.101872. Epub 2022 Nov 7. PMID: 36595951; PMCID: PMC9637521.



Chapter 12

Directed Differentiation of Human Pluripotent Stem Cells for the Generation of High-Order Kidney Organoids

Idoia Lucía Selfa, Maria Gallo, Nuria Montserrat, and Elena Garreta

Abstract

Our understanding in the inherent properties of human pluripotent stem cells (hPSCs) have made possible the development of differentiation procedures to generate three-dimensional tissue-like cultures, so-called organoids. Here we detail a stepwise methodology to generate kidney organoids from hPSCs. This is achieved through direct differentiation of hPSCs in two-dimensional monolayer culture toward the posterior primitive streak fate, followed by induction of intermediate mesoderm-committed cells, which are further aggregated and cultured in three-dimensions to generate kidney organoids containing segmented nephron-like structures in a process that lasts 20 days. We also provide a concise description on how to assess renal commitment during the time course of kidney organoid generation. This includes the use of flow cytometry and immunocytochemistry analyses for the detection of specific renal differentiation markers.

Key words Kidney organoid, Human pluripotent stem cells, Differentiation, Primitive streak, Intermediate mesoderm, Nephron progenitor cells, 2D Monolayer, 3D Organotypic culture, Nephrons, Flow cytometry, Immunocytochemistry

1 Introduction

Human pluripotent stem cells (hPSCs) can be exposed to a series of developmental cues in form of cytokines, growth factors, chemical compounds, and biophysical cues [1] to direct their differentiation toward specific cell lineages occurring during kidney development [2]. Under these renal inductive signals, differentiating hPSCs are able to self-organize in three-dimensional (3D) culture resulting in the formation of tissue-like structures that partially resemble in structure and function the human developing kidney, known as kidney organoids (reviewed in [3–5]). Several studies have described different approaches to generate hPSC-derived kidney organoids containing nephron-like structures [1, 6–9]. Most of the procedures developed up to date account with a first stage of

differentiation in where undifferentiated hPSCs are guided to the posterior primitive streak (PPS) fate by the endogenous activation of WNT using the GSK-3 β inhibitor CHIR99201 (CHIR). To that end, different lengths and doses of CHIR treatment have been described [1, 6–11]. In the same manner, different laboratories have shown that the commitment of PPS toward intermediate mesoderm (IM) and nephron progenitor cells (NPCs) can vary among protocols [1, 6–11]. In this chapter, we explain in detail a procedure to generate kidney organoids from hPSCs, during which hPSCs are induced for a short period of 4 days in a two-dimensional (2D) culture fashion toward IM-like cells that are then aggregated into 3D spheroids and cultured under organotypic conditions for 16 additional days (Fig. 1). After aggregation, IM-committed spheroids are kept in the presence of growth factors for 5 days to induce NPC commitment. Three additional days are then required for the formation of renal vesicle (RV) structures—the precursor structures of the nephrons—that appear within the organoids 24 h after complete growth factor removal due to a process of mesenchymal to epithelial transition. During the following 8 days, RVs acquire proximal–distal polarity and develop into nephron-like structures by recapitulating in vitro the process known as nephron patterning (Fig. 1). Day 16 kidney organoids reveal the presence of segmented nephron-like structures containing glomeruli with podocyte-like cells connected to proximal and distal tubular structures [1]. In contrast to recent works that showed the possibility to differentiate hPSCs into kidney organoids transcriptomically matching first trimester gestational kidney in a process of 25 days, the method described here takes advantage of the aggregation of IM-like cells very early during the differentiation process to boost cell-to-cell and cell-to-extracellular matrix interactions to efficiently generate kidney organoids with a similar transcriptomic profile as that found in second-trimester human gestational kidney [1].

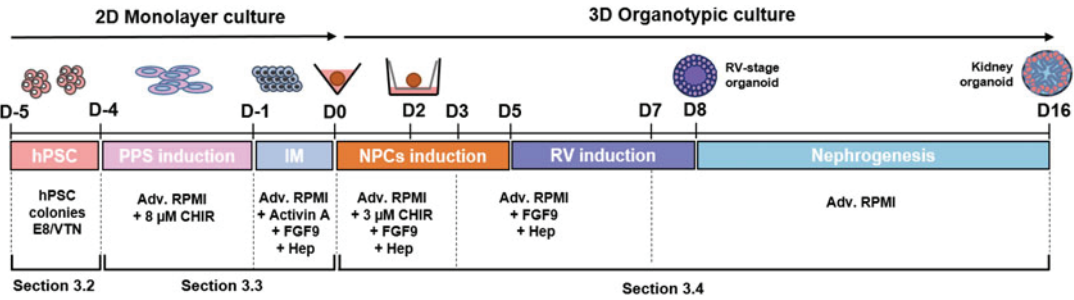


Fig. 1 Kidney organoid differentiation protocol. The days of the protocol are indicated as “D”. *hPSCs* human pluripotent stem cells, *PPS* posterior primitive streak, *IM* intermediate mesoderm, *NPCs* nephron progenitor cells, *RV* renal vesicles, *E8* essential 8 medium, *VTN* vitronectin, *Adv RPMI* advanced RPMI 1640 basal medium, *Hep* heparin

During the last decade, several laboratories have demonstrated the generation of organoids from hPSCs resembling not only kidney but also a variety of organs including eye cup, brain, intestine, and lung among others [5]. The field is rapidly evolving, and several studies have recently highlighted organoids' potentiality for modeling organ development and disease in the human context, opening the door to future drug discovery and regenerative medicine applications. However, the organoid field is still facing major challenges, in part associated to the lack of control in the self-organizing events occurring during organoid formation in current methodologies, leading to high organoid variability as well as lack of essential cellular components (i.e., vascularization) and incomplete maturation. In this regard, emergent bioengineering technologies including biomimetic materials, microtechnologies, and 3D bioprinting [12, 13] can be used to harness and control organoid morphogenesis by precisely tuning the organoid niche (i.e., cell-cell and cell-extracellular matrix interactions) (reviewed in [5, 14]). Recently, we have shown that kidney organoids can vascularize upon implantation onto the chick chorioallantoic membrane (CAM), thus acquiring relevant features of podocyte maturity [1]. Of note, when the soft CAM microenvironment was mimicked in vitro using polyacrylamide hydrogels, these accelerated the formation of kidney organoids that indeed contained more nephron-like structures in comparison to a rigid microenvironment [1]. Overall, the exploitation of bioengineering tools as well as other emergent disciplines (i.e., gene editing, single-cell analysis, force mapping, computational modeling, among others) in combination with the morphogenetic potential of organoids represents a promising scenario toward the development of next-generation organoid models.

2 Materials

2.1 Culture and Passage of hPSCs

1. Air-vented 10 cm Petri dishes, Nunclon Delta™ (150350, Thermo Scientific).
2. Phosphate-buffered saline (PBS) pH 7.4 (1×) (1001–015, Life Technologies).
3. Vitronectin (VTN-N) (A14700, Fisher Scientific). The vitronectin coating is prepared by diluting the vitronectin stock solution (0.5 mg/mL) at a 1:100 ration in 1× PBS. Coating of an air-vented 10 cm Petri dish is performed by diluting 50 µL of the vitronectin stock solution in 5 mL of PBS to have a final concentration of 0.5 µg of vitronectin/mL. For 24-well plates (*see* Subheading 2.2), 400 µL of diluted vitronectin (5 µg/mL) per well is used. Plates containing the vitronectin solution are left at room temperature for 1 h or kept at 4 °C overnight until further use.

4. Essential 8 medium (A1517001, Life Technologies). It is provided as a two-component kit (500 mL basal medium bottle and 10 mL supplement). Besides adding the 10 mL supplement, add 5 mL of Penicillin/Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin, 15140122, Life Technologies).
5. 0.5 M EDTA (15575-038, Life Technologies): dilute the EDTA stock solution (0.5 M) at a 1:1000 ratio in 1× PBS to have a working solution of 0.5 mM EDTA.

2.2 Plating of hPSCs for Differentiation

1. 24-well plate, Nunclon Delta™ (142475, Thermo Scientific).
2. Round coverslips, diameter 12 mm, #1.5 (CBA-D00120RAC20MNZ#0, Fischer Scientific). Autoclave the coverslips before use for cell culture.
3. Microfuge tubes, 1.5 mL (200400P, Deltalab).
4. Accumax (07921, Stem Cell Technologies).
5. Dulbecco's modified eagle medium (DMEM) (11966025, Life Technologies).
6. Fetal Bovine Serum (FBS) (10270-106, Gibco).
7. Neubauer chamber (0640010, Superior Marienfeld).

2.3 Differentiation of hPSCs Toward IM-Committed Cells and Generation of Kidney Organoids

1. Advanced RPMI 1640 basal medium (12633-012, Life Technologies): A bottle of 500 mL of advanced RPMI 1640 basal medium is supplemented with 5 mL of L-GlutaMAX (200 mM, 35050-038, Life Technologies) and 5 mL of Penicillin/Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin, 15140122, Life Technologies).
2. 12 µM CHIR99021 (SML1046-5MG, Sigma): dilute 5 mg of CHIR99021 in 896 µL of dimethyl sulfoxide (D2650, Sigma), aliquot, and store at −20 °C.
3. 50 µg/mL Recombinant human FGF9 (100-23B, Peprotech): reconstitute in cell-culture-grade distilled water (15230-089, Life Technologies), aliquot, and store at −20 °C.
4. 50 mg/mL Heparin (H3149-10KU, Sigma): reconstitute in cell-culture-grade distilled water (15230-089, Life Technologies), aliquot, and store at 4 °C.
5. 50 µg/mL Activin A (338-AC-050, R&D Systems): reconstitute in sterile 4 mM hydrochloric acid (HCl), aliquot, and store at −20 °C.
6. 96-well plate, V-bottom, Nunc™ (249935, Thermo Scientific).
7. Corning® Transwell® polyester membrane cell culture inserts (CLS3460, Sigma).
8. Thin glass Pasteur pipette, 150 mm (5426015, Normax).

9. 200 μ L-wide orifice pipette tips (E1011-8400, Starlab). Tips need to be autoclaved for cell culture use.

**2.4 Flow Cytometry
Analysis
of Differentiation
Markers**

1. eBioscience™ Fcγ3/Transcription factor staining buffer set (00-5523-00, Invitrogen). Components are fixation/permeabilization concentrate (00-5123), fixation/permeabilization diluent (00-5223) and 10× permeabilization buffer (00-8333). Prepare fresh Fcγ3 fixation/permeabilization working solution by mixing one part of Fcγ3 fixation/permeabilization concentrate with three parts of Fcγ3 fixation/permeabilization diluent. Prepare a 1× working solution of permeabilization buffer by mixing one part of 10× permeabilization buffer with nine parts of distilled water.
2. Accumax (07921, Stem Cell Technologies).
3. Falcon® 5 mL Round-Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap (352235, Corning).
4. LIVE/DEAD™ Fixable violet dead cell stain kit (L34963, Invitrogen).
5. Fetal bovine serum (FBS) (10270-106, Gibco).

Antibodies and Isotype Controls

6. Oct3/4, mouse IgG1 κ , Alexa Fluor (AF) 488, Clone 40/Oct3 (560253, BD Pharmingen).
7. Mouse IgG1 κ , AF488-conjugated isotype control, clone MOPC-21 (557721, BD Pharmingen).
8. Brachyury, goat IgG, APC (IC2085A, R&D Systems).
9. Goat IgG, APC-conjugated isotype control (IC108A, R&D Systems).
10. PAX2, goat IgG (AF3364, R&D Systems). Before use, conjugate PAX2 antibody to AF488 using the Lightning-Link® Rapid conjugation kit (322-0010, Innova Biosciences) following manufacturer instructions.
11. Goat IgG, AF488-conjugated isotype control (IC108G, R&D Systems).

**2.5 Immuno-
cytochemistry
Analysis
of Differentiation
Markers**

1. Fixation solution of 4% paraformaldehyde. In the fume hood, prepare this solution by mixing 2.5 mL of 16% paraformaldehyde (153799, Anane) with 7.5 mL of 1× PBS. Prepare fresh and use it upon preparation.
2. Tris-buffered saline (TBS), 10× (pH 7.4–7.5). To prepare this buffer, dissolve 132.2 g of Trizma-HCl (T6666, Sigma), 19.4 g of Trizma base (T6791, Sigma), and 90.0 g of NaCl (S7653, Sigma) in 855 mL of distilled water. Keep the solution at 4 °C and use it within 1 month.

3. TBS, 1× (pH 7.4–7.5). To prepare this buffer, dilute 10× TBS at 1:10 ratio by mixing 900 mL of distilled water and 100 mL of 10× TBS. Keep the solution at 4 °C and use it within 2 weeks.
4. TBS, 1× (pH 7.4–7.5) supplemented with 1% Triton X-100 (50 mL). To prepare this buffer, dilute 500 µL of Triton X-100 (T8787, Sigma) in 50 mL of TBS, 1× (pH 7.4–7.5). Keep the solution at 4 °C and use it within 2 weeks.
5. TBS, 1× (pH 7.4–7.5) supplemented with 0.5% Triton X-100 (50 mL). To prepare this buffer, dilute 250 µL of Triton X-100 (T8787, Sigma) in 50 mL of TBS, 1× (pH 7.4–7.5). Keep the solution at 4 °C and use it within 2 weeks.
6. Blocking solution (10 mL) containing 1% Triton X-100 and 6% donkey serum. To prepare this solution, dilute 600 µL of donkey serum (S30-KC, Sigma) in 9.4 mL of TBS, 1× (pH 7.4–7.5) containing 1% Triton X-100. Prepare fresh blocking solution and use it upon preparation.
7. Streptavidin/Biotin blocking kit (SP-2002, Vector Labs) is used as an additional blocking step when biotinylated Lotus Tetragonolobus Lectin (LTL) is used to stain proximal tubule-like structures. Briefly, use 4–5 drops of streptavidin solution and incubate 20 min. Wash once with 1× TBS for 15 min at RT. Then use 4–5 drops of biotin solution and incubate 20 min. Wash once with 1× TBS for 15 min at RT.
8. For diluting the antibodies and for the washing steps, prepare a solution (25 mL) of 1× TBS containing 0.5% Triton X-100 and 6% donkey serum by mixing 1500 µL of donkey serum (S30-KC, Sigma) in 23.5 mL of 1× TBS containing 1% Triton X-100. Prepare fresh and use it upon preparation.
9. In case LTL is used to stain proximal tubule-like structures, for diluting the antibodies and LTL, and for the washing steps, prepare a solution (25 mL) of 1× TBS containing 0.5% Triton X-100 and 1% bovine serum albumin (BSA) by dissolving 0.25 g of BSA (A4503, Sigma) in 25 mL of 1× TBS containing 0.5% Triton X-100. Prepare fresh and use it upon preparation.

2.6 Primary Antibodies

1. Brachyury, goat IgG (AF2085, R&D Systems).
2. Oct4, mouse IgG, clone C10 (Sc-5279, Santa Cruz Biotechnology).
3. PAX2, goat IgG (AF3364, R&D Systems).
4. SALL1, mouse IgG2a, clone K9814 (PP-K9814-00, R&D Systems).
5. SIX2, rabbit IgG (11562-1-AP, Proteintech).

6. WT1, rabbit IgG, clone CAN-R9(IHC)-56-2 (ab89901, Abcam).
7. E-Cadherin, mouse IgG2a, κ , clone 36 (610181, BD Bioscience).
8. Podocalyxin, goat IgG (BAF1658, R&D Systems).

2.7 Secondary Antibodies and Other Reagents

1. Donkey anti-goat IgG AF488 (705-545-147, Jackson ImmunoResearch).
2. Donkey anti-mouse IgG Cy3 (715-165-151, Jackson ImmunoResearch).
3. Donkey anti-mouse IgG AF488 (A21202, Fischer Scientific).
4. Donkey anti-rabbit IgG Cy3 (711-165-152, Jackson ImmunoResearch).
5. Donkey anti-goat IgG AF555 (A21432, Fischer Scientific).
6. Donkey anti-mouse IgG AF647 (715-605-151, Jackson ImmunoResearch).
7. Biotinylated Lotus Tetragonolobus Lectin (LTL) (B-1325, Vector Laboratories).
8. DyLight 488 Streptavidin (SA-5488, Vector Labs).
9. 4',6-Diamidino-2-Phenylindole, dihydrochloride (DAPI) (D1306, Invitrogen). To make a 5 mg/mL (14.3 mM) DAPI stock solution, dissolve the contents of one vial (10 mg) in 2 mL of distilled water. For long-term storage, aliquot the stock solution and store at -20°C .

2.8 Other Materials

1. Histology mold, plastic (20447200820, Laboquimia). To embed the organoids, prepare a solution of 0.8% low gelling temperature type VII agarose (A4018, Sigma) by dissolving 0.8 g of agarose in 100 mL of distilled water. Use the plastic mold to place the organoids together with pre-warmed 0.8% agarose. Leave at 4°C to obtain an agarose block containing the organoids.
2. Dako Pen (S200230, Agilent). Use it to surround tissue sections on the slide to create a smaller area for antibody incubation.
3. Fluoromount-G (0100-01, Southern Biotech).
4. Round coverslips, diameter 12 mm, #1.5 (CBA-D00120RAC20MNZ#0, Thermo Scientific).
5. Rectangular coverslip, 24×50 mm, #1.5 (BBAD02400500#SC13MNZ#0##, Thermo Scientific).
6. Microscope slide, $25 \times 75 \times 1.0$ mm (J1800AMNZ, Thermo Scientific).
7. Nail polish to fix the coverslips when mounting the samples.

3 Methods

The methodology to generate kidney organoids from hPSCs (Fig. 1) has been divided into four methodological stages involving the culture and passage of hPSCs (Subheading 3.1), the plating of hPSCs for differentiation (Subheading 3.2), the differentiation of hPSCs toward posterior primitive streak (PPS) and intermediate mesoderm (IM) (Subheading 3.3), and the formation and culture of kidney organoids (Subheading 3.4). Next, by means of flow cytometry (Subheading 3.5) and immunocytochemistry (Subheading 3.6) analyses, a methodology to analyze the differentiation outcomes at different time points during the generation of kidney organoids is also described.

3.1 Culture and Passage of hPSCs

1. hPSCs are grown on vitronectin-coated air-vented 10 cm Petri dishes in Essential 8 Medium and incubated with 5% CO₂ at 37 °C. In order to avoid their spontaneous differentiation, hPSCs need to be passaged before they reach 100% confluency.
2. For enzymatic passaging, aspirate the Essential 8 medium from hPSCs monolayers at approximately 80% confluency.
3. Perform a quick and gentle wash by rinsing twice with 8 mL of 1x PBS.
4. Add 5 mL of 0.5 mM EDTA. Place the cells in an incubator at 37 °C for 3–4 min. Gently aspirate the EDTA solution without disrupting the hPSC colonies. Then disaggregate the hPSC colonies by flushing 1 mL of Essential 8 media to the hPSC colonies in order to detach the cells into small clusters (*see Note 1*). Collect the clumps of cells into a tube. To prevent excessive dissociation of cells, use a fresh 1 mL of Essential 8 Media each time and repeat the process until complete detachment of all cells in the plate. Normally, 10 mL of Essential 8 Media is used to collect the cell clumps in a 15-mL tube.
5. Take 500 µL of the cell suspension and complete to 10 mL with Essential 8 media in a new 15-mL tube to passage the cells at a 1:20 ratio (*see Note 2*).
6. Aspirate the vitronectin of a new air-vented 10 cm Petri dish (for vitronectin coating preparation, *see Subheading 2.1, item 3*) and directly plate the diluted cell suspension. Gently move the plate in all directions to evenly distribute the cell clusters on the plate.
7. Culture the cells in an incubator at 37 °C with 5% CO₂ for approximately 6 days, changing the Essential 8 media every second day (*see Note 2*).

3.2 Plating of hPSCs for Differentiation

1. Prepare fresh 24-well plates coated with vitronectin. Prepare also an additional 24-well plate containing 12 mm round glass coverslips coated with vitronectin, which will be further used to analyze the extent of differentiation into PPS and IM by immunocytochemistry (*see* Subheading 3.6).
2. Start the procedure as if it is a normal hPSCs passage and collect the undifferentiated cells in 10 mL of Essential 8 media in a 15-mL tube (*see* Subheading 3.1, steps 1–4).
3. For cell counting, take 1 mL of the cell suspension, place it in a 1.5 mL microfuge tube, and centrifuge for 5 min at 100 *g* to obtain a cell pellet (*see* **Note 3**).
4. Remove the Essential 8 media, add 1 mL of 1× PBS to wash the cell pellet, and centrifuge again for 5 min at 100 *g*.
5. Remove the 1× PBS and add 300 μ L of Accumax and incubate at 37 °C for 5 min to allow single-cell dissociation for cell counting.
6. After the incubation time, cancel Accumax activity by adding 700 μ L of DMEM media supplemented with 10% FBS.
7. Take 10 μ L of the single-cell suspension and place it in a Neubauer chamber for cell counting (*see* **Note 4**).
8. Based on cell number, resuspend the cells in the appropriate volume of Essential 8 media to have 2×10^5 cells/mL. Plate 500 μ L of the resultant cell suspension per well of a 24-well plate to have 10^5 cells in each well (*see* **Note 5**).
9. Plating density and cell colony distribution is essential for an efficient differentiation. In order to prevent cell clusters to come together in the center of the wells of a 24-well plate, shake the plate in all directions and carefully place the plate in the incubator. Avoid opening and closing the incubator during the next 2 h to ensure an even distribution of the adhered cells in the plate.
10. Culture the cells at 37 °C with 5% CO₂ for 24 h before starting the differentiation (Fig. 2a, b; *see* **Note 6**) (Fig. 1, day –5).

3.3 Differentiation of hPSCs Toward Posterior Primitive Streak (PPS) and Intermediate Mesoderm (IM)

1. Once cell colony density and distribution are adequate (Fig. 3a), aspirate the Essential 8 media from the 24-well plate and rinse once with 1× PBS to remove remnants of it (Fig. 1, day –4).
2. Add 500 μ L/well of advanced RPMI 1640 basal media supplemented with 8 μ M CHIR (Fig. 1, day –4).
3. Every 24 h, replace the media with fresh advanced RPMI 1640 basal media supplemented with 8 μ M CHIR for two additional consecutive days (Fig. 1, day –3 and day –2). Note that hPSCs start to change their morphology (Fig. 3b).

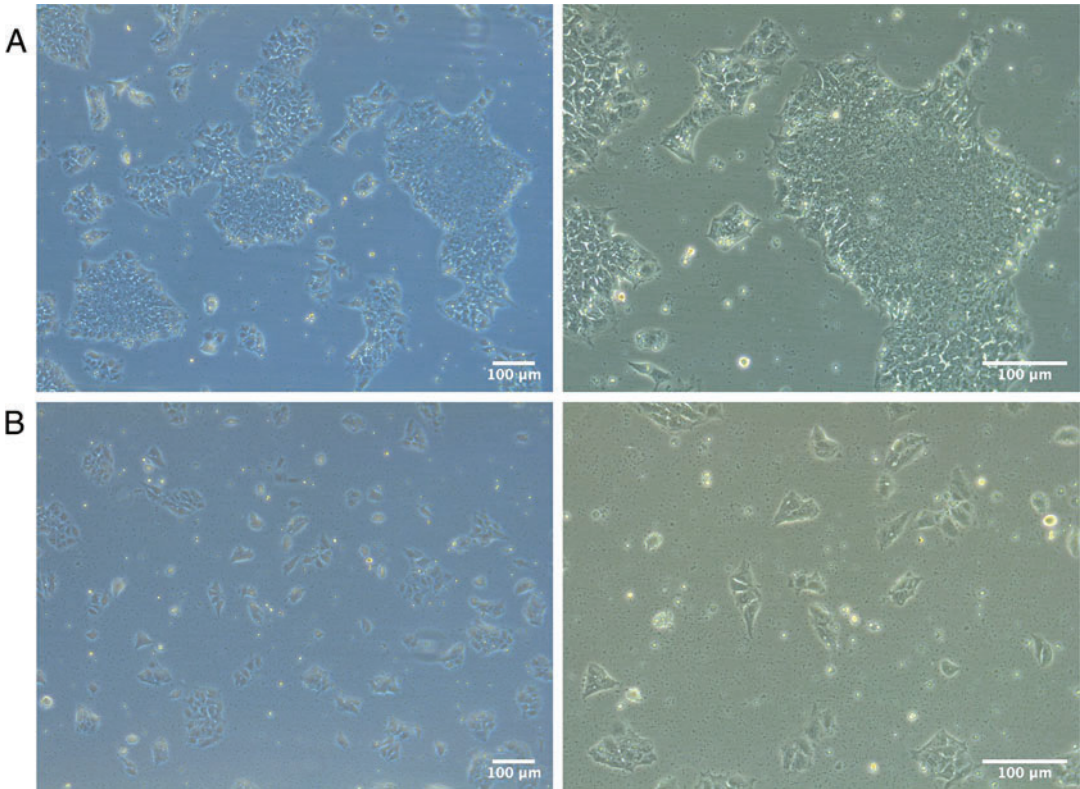


Fig. 2 Optimal plating density and colony distribution of undifferentiated hPSCs cultured in Essential 8 media that is required for starting the differentiation. Representative bright-field images of ES[4] hPSCs 24 h after plating (day -4). **(a)** Differentiation is started from hPSCs colonies presenting a good compaction. **(b)** When hPSCs colonies are too small or are not well compacted, wait 24 h more before starting the differentiation. If waiting 24 h does not improve hPSCs colony compaction or the right confluency is not achieved, then repeat the plating of hPSCs. Higher magnification images ($10\times$) on the right correspond to the images shown on the left ($5\times$). Scale bars: 100 μm

4. After the 3 days of 8 μM CHIR treatment (Fig. 1, day -1), cultured cells show an appearance of dense clusters (Fig. 3c). In order to confirm that cells have started to acquire a PPS-related fate, the differentiation extent is measured at the molecular level by the collection of cells and analysis for the loss of pluripotency-related markers such as OCT4, and the acquisition of the primitive streak marker Brachyury through both flow cytometry (*see* Subheading 3.5) and immunocytochemistry analysis (Fig. 4a; *see* Subheading 3.6).
5. On day -1 , remove the advanced RPMI 1640 basal media supplemented with 8 μM CHIR and gently rinse once with $1\times$ PBS (*see* Note 7).

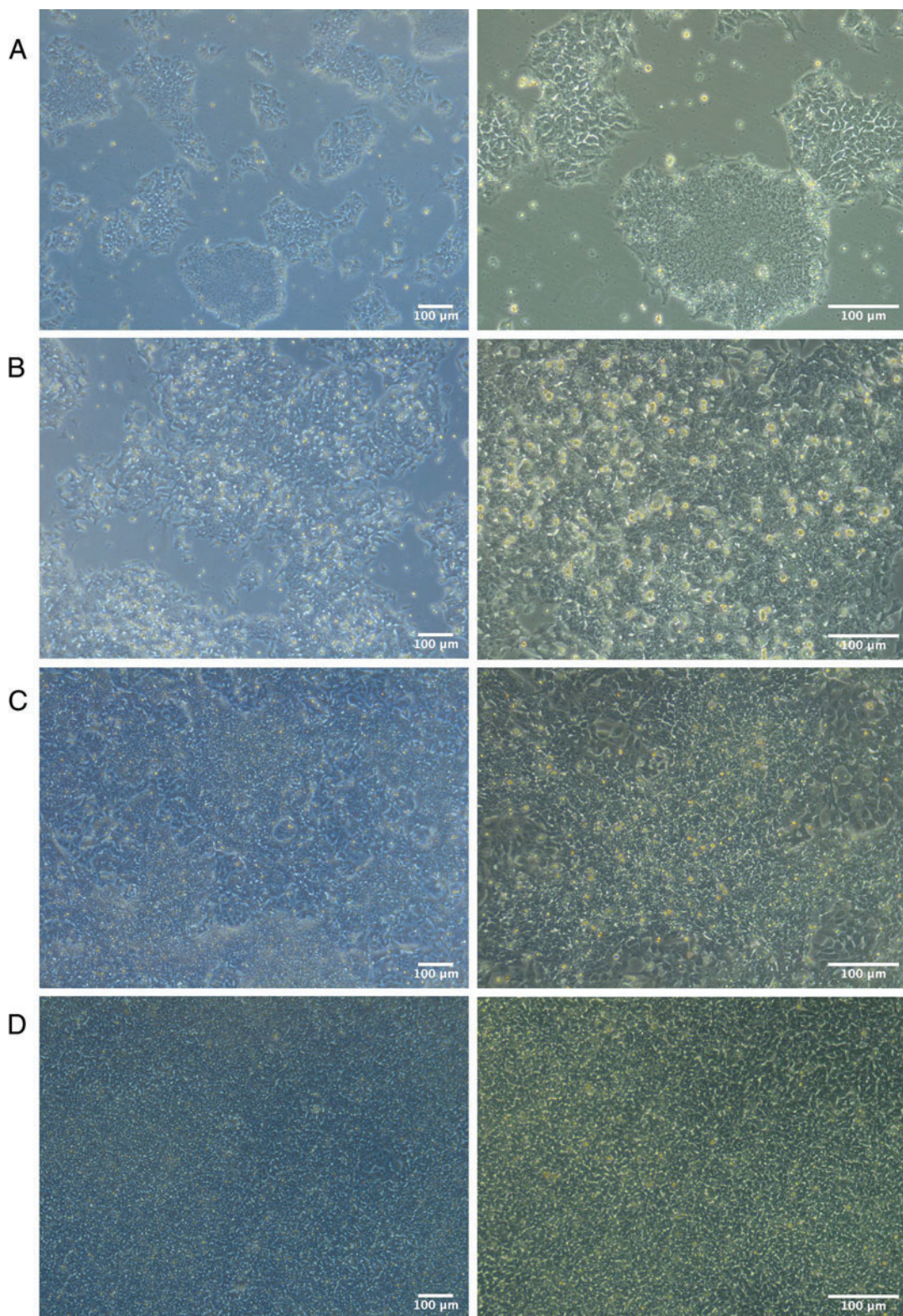


Fig. 3 Morphological changes in hPSCs during the first 4 days of 2D monolayer differentiation. Representative bright-field images. (a) Day -4: ES[4] hPSCs 24 h after plating that show the optimal confluency (40–50%) and morphology needed for starting the 8 μ M CHIR treatment. (b) Day -2: morphology of differentiating hPSCs after 48 h of 8 μ M CHIR treatment. (c) Day -1: appearance of cells after 72 h of

- 6. Add 500 μ L per well of advanced RPMI 1640 basal media supplemented with 200 ng/mL FGF9, 1 μ g/mL heparin, and 10 ng/mL activin A (Fig. 1, day -1).
- 7. After 24 h (Fig. 1, day 0), cells should appear under the microscope as a tight monolayer (Fig. 3d).
- 8. Cells at this point can be analyzed for the expression of the IM marker PAX2, using both flow cytometry (*see* Subheading 3.5) and immunocytochemistry analysis (Fig. 4b; *see* Subheading 3.6).

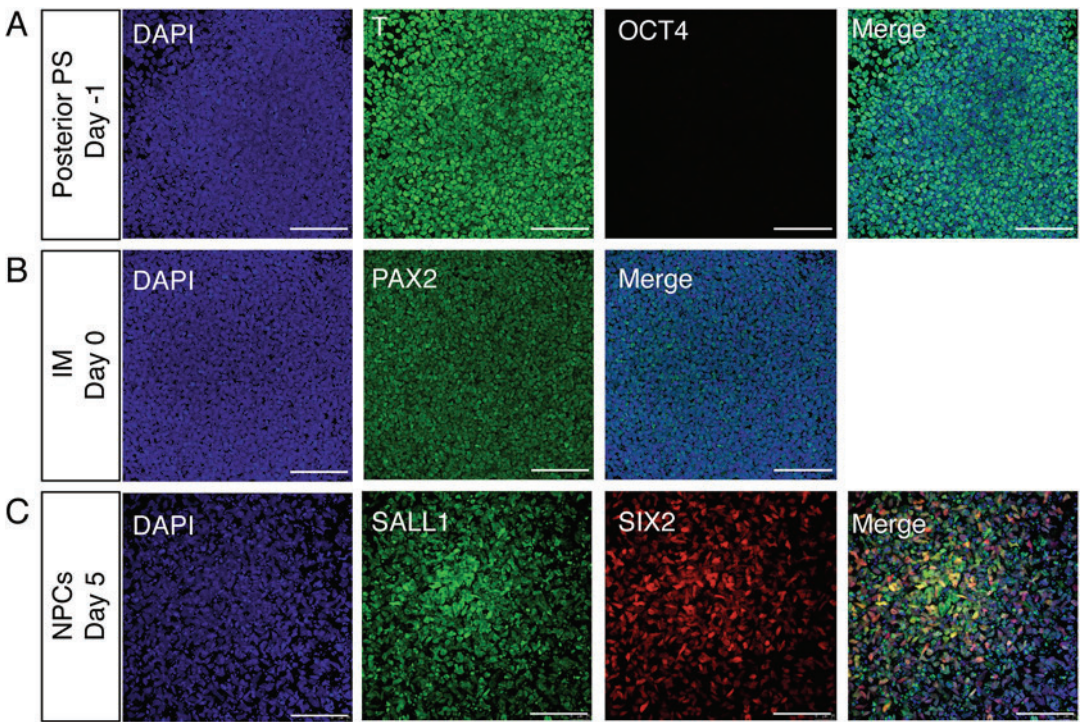


Fig. 4 Immunocytochemistry analysis for the assessment of the differentiation extent of monolayer cell cultures at different time points during the differentiation protocol. (a) Immunocytochemistry is performed at day -1 to detect the expression of Brachyury (T), one of the major markers related to PPS identity. Notice that T-positive cells do not express OCT4, one of the core pluripotency-related markers. (b) The commitment of PPS toward the IM is assessed by the detection of PAX2 at day 0. (c) The NPC signature in day 5 cells is assessed by the detection of SALL1 and SIX2 markers. Scale bars: 100 μ m

← **Fig. 3** (continued) 8 μ M CHIR treatment that induces the PPS fate. A compact cell monolayer is starting to form, but still loose clusters of cells are found at this stage. (d) Day 0: appearance of cells after treatment with FGF9, activin A, and heparin for 24 h that induces the derivation of intermediate mesoderm-committed cells. The cell monolayer appears uniform and compacted. At this stage, monolayers are ready to aggregate into 3D organoids. Higher magnification images (10 $\times$) on the right correspond to the images shown on the left (5 $\times$). Scale bars: 100 μ m

3.4 Formation and Culture of Kidney Organoids

1. On day 0, remove the media and rinse twice with $1\times$ PBS. Place 500 μL of Accumax per well of the 24-well plate and incubate for 1 min at 37°C . Carefully, aspirate the Accumax without disrupting the cell monolayer. Then, use 500 μL of fresh advanced RPMI 1640 basal media to dissociate the cell monolayer in each well and collect the single-cell suspension in a 15-mL tube (*see Note 8*). Normally, 12 mL of advanced RPMI 1640 basal media is used to collect the cells from a 24-well plate.
2. Usually, since large amounts of cells are recovered after cell dissociation, an aliquot of the cell suspension is diluted at 1:4 ratio in advanced RPMI 1640 basal media to have a proper cell density to correctly perform the cell counting. Then, 10 μL of the single-cell suspension are placed in a Neubauer chamber and cells are counted (*see Note 3*).
3. Based on cell number, resuspend the cells in the appropriate volume of advanced RPMI 1640 basal media supplemented with 3 μM CHIR, 200 ng/mL FGF9, and 1 $\mu\text{g/mL}$ heparin to have 5×10^6 cells/mL. Pipette 150 μL of the cell suspension in each well of a 96-well plate (V-bottom) to have 5×10^5 cells per well (*see Note 9*).
4. Centrifuge the 96-well plate (V-bottom) for 3 min at 300 g (Fig. 1, day 0).
5. Maintain the 96-well plate in an incubator at 37°C with 5% CO_2 for 48 h without medium change to allow cells to self-aggregate and induce the differentiation toward nephron progenitor cells (NPCs).
6. After 48 h (day 2), the self-aggregated spheroids are transferred to 12-well plate transwells. Carefully transfer one spheroid per transwell by placing the spheroid on top of the transwell membrane (*see Note 10*).
7. Immediately, add 450 μL of advanced RPMI 1640 basal media with 3 μM CHIR, 200 ng/mL FGF9, and 1 $\mu\text{g/mL}$ heparin to the base of each transwell to generate an air-liquid interface organotypic culture condition (Fig. 1, day 2).
8. On day 3, aspirate the media from the transwells with thin glass Pasteur pipettes and replace it with 450 μL of advanced RPMI 1640 basal media containing only 200 ng/mL FGF9 and 1 $\mu\text{g/mL}$ heparin, and culture for 48 h without media changes.
9. On day 5, aspirate the media from the transwells with thin glass Pasteur pipettes and replace it with 450 μL of fresh advanced RPMI 1640 basal media containing 200 ng/mL FGF9 and 1 $\mu\text{g/mL}$ heparin, and culture for 48 h without media changes. At this point, NPCs induction can be assessed by analyzing the expression of NPC-associated markers by immunocytochemistry (Fig. 4c, *see Subheading 3.6*).

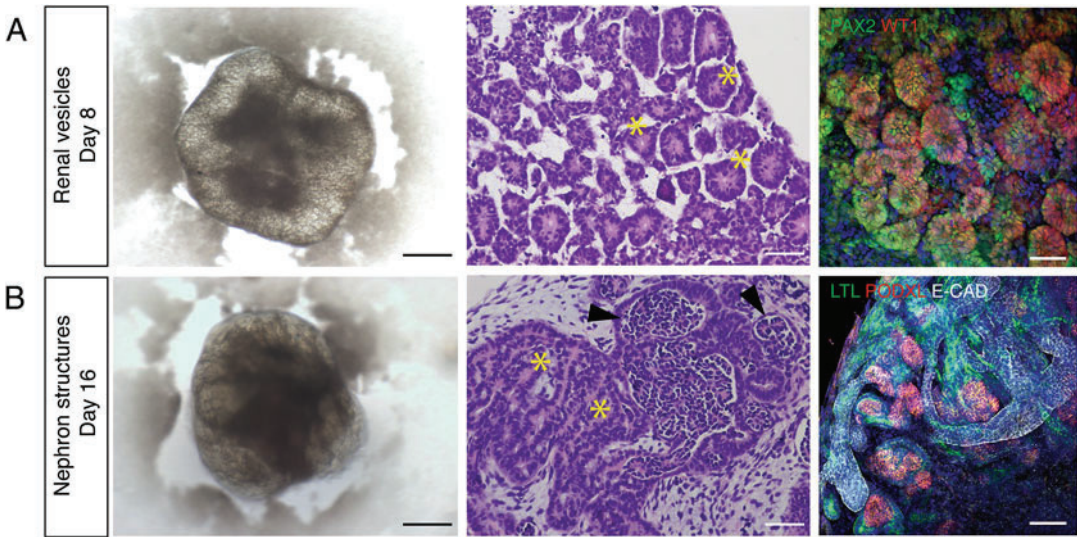


Fig. 5 Characterization of renal vesicles (RVs) and segmented nephron-like structures in hPSC-derived kidney organoids. **(a)** RV-stage kidney organoids contain RV-like structures that can be detected by different techniques. From left to right: bright-field image of a RV-stage kidney organoid, hematoxylin-eosin staining on a paraffin section of a RV-staged kidney organoid, and immunocytochemistry for the detection of PAX2 and WT1 in a RV-stage kidney organoid at day 8 of differentiation. Asterisks point to renal vesicle structures. **(b)** Segmented nephron-like structures are analyzed at day 16 of differentiation by different techniques. From left to right: bright-field image of a day 16 kidney organoid, hematoxylin-eosin staining on a paraffin section of a day 16 kidney organoid, and immunocytochemistry for the detection of Podocalyxin (PODXL) in podocyte-like cells, and Lotus Tetragonolobus Lectin (LTL) and E-cadherin (ECAD) in proximal and distal segments of the tubular-like structures, respectively. Arrowheads point to glomerular-like structures, and asterisks point to tubular-like structures. Scale bars in bright-field images correspond to 500 μm . Scale bars in hematoxylin-eosin staining correspond to 50 μm . Scale bars in immunocytochemistry staining correspond to 75 μm

10. On day 7, growth factors are removed by replacing the media with advanced RMPI 1640 basal media. After 24 h, multiple renal vesicles (RVs)—the precursor structures of the nephrons—clearly appear within the spheroid (Fig. 1, day 8 RV-stage organoid). RVs can be visualized in bright field, and further analyzed by performing hematoxylin and eosin staining of kidney organoid sections and immunocytochemistry for the expression of RV-associated markers (Fig. 5a; see Subheading 3.6).
11. Change the media every second day until day 16, when kidney organoids are fully developed and contain nephron-like structures (Fig. 1, day 16 kidney organoid). The nephron structures can be visualized in bright field and further analyzed by performing hematoxylin and eosin staining of kidney organoid sections and immunocytochemistry for the expression of nephron markers (Fig. 5b; see Subheading 3.6).

3.5 Flow Cytometry Analysis of Differentiation Markers

1. For analysis of PPS differentiation efficiency, harvest cells at day -1 . PPS induction is characterized by a marked decrease in the expression of the pluripotency-associated marker OCT4 and upregulation of the primitive streak marker Brachyury. For analysis of IM differentiation efficiency, harvest cells at day 0. IM commitment is assessed by analyzing the expression of PAX2.
2. For intracellular staining of all the above markers, use the Foxp3/transcription factor staining buffer set (*see* Subheading 2.4 for buffer preparation).
3. A minimum number of 10^5 cells/tube should be used for the analysis hereafter. Therefore, harvest approximately 3–4 wells of the 24-well plate at day -2 and 2–3 wells of the 24-well plate at day -1 , to perform the analysis.
4. Remove media and gently rinse twice with $1 \times$ PBS.
5. Add 300 μ L/well of Accumax and incubate for 2–5 min at 37 °C.
6. Aspirate the Accumax, dissociate cells by flushing with $1 \times$ PBS, and collect them in $1 \times$ PBS. Collect the necessary wells and resuspend the cells in 1 mL of $1 \times$ PBS.
7. Pass the cell suspension to a flow cytometry tube with 35- μ m-filter caps to ensure a single-cell suspension (*see* Subheading 2.4, item 3).
8. Add 1 μ L of reconstituted LIVE/DEAD fixable violet stain (*see* **Note 11**; reconstituted reactive should be used in 2 weeks) and incubate for 30 min at room temperature (RT) in the dark.
9. Wash two times with 3 mL of $1 \times$ PBS and centrifuge for 3 min at 300 g . Discard the supernatant and pulse vortex the sample to completely dissociate the pellet.
10. Add 500 μ L of Foxp3 fixation/permeabilization working solution to each tube and resuspend the cells in the solution.
11. Incubate for 30 min at RT in the dark.
12. Centrifuge the sample for 3 min at 300 g and discard the supernatant.
13. For permeabilization, wash two times with 3 mL of permeabilization buffer. Centrifuge the sample for 3 min at 300 g and discard the supernatant.
14. Resuspend the pellet with permeabilization buffer. At this point, divide the sample into the necessary tubes to perform the isotype control staining and the correspondent antibody stainings. For PPS analysis (day -1), resuspend in 2.5 mL of permeabilization buffer and pipette 500 μ L of cell suspension per tube (five tubes). For IM analysis (day 0), resuspend in

1.5 mL of permeabilization buffer and pipette 500 μ L of cell suspension per tube (three tubes).

15. Block the sample tubes with 2% FBS by adding 10 μ L directly to each tube containing 500 μ L of the cell suspension. Incubate for 15 min at RT.
16. Centrifuge the tubes for 3 min at 300 g and discard the supernatant by gently pouring the solution without disrupting the pellet.
17. Add 100 μ L of permeabilization buffer to each tube and pulse vortex to dissociate the pellet.
18. Add the recommended amount of conjugated isotype controls and antibodies for detection of intracellular antigens to each tube, pulse vortex and incubate for 30 min at RT in the dark.
19. For PPS analysis (day -1), add the indicated isotype controls and antibodies:
 - (a) Tube 1: do not add any reagent.
 - (b) Tube 2: Goat IgG APC-conjugated isotype control (10 μ L) + Mouse IgG1 AF488-conjugated isotype control (5 μ L).
 - (c) Tube 3: Brachyury-APC (10 μ L).
 - (d) Tube 4: Oct4-AF488 (20 μ L).
 - (e) Tube 5: Brachyury -APC (10 μ L) + Oct4-AF488 (20 μ L).
20. For IM analysis (day 0), add the indicated isotype controls and antibodies:
 - (a) Tube 1: do not add any reagent.
 - (b) Tube 2: Goat IgG AF488-conjugated isotype control (5 μ L).
 - (c) Tube 3: PAX2-AF488 (0.5 μ L).
21. After incubation, add 1 mL of permeabilization buffer to each tube and centrifuge the tubes for 5 min at 300 g . Discard the supernatant.
22. Wash two times with 2 mL of permeabilization buffer to each tube, centrifuging the tubes for 5 min at 300 g and discard the supernatant.
23. Resuspend each cell pellet in 500 μ L of $1 \times$ PBS supplemented with 2% FBS.

Acquire the sample tubes in a flow cytometer and analyze the data using a flow cytometry software such as FlowJo. The expected percentage of Brachyury⁺ OCT4⁻ cells in the PPS analysis (day -1) is around 80%. The expected percentage of PAX2⁺ cells in the IM analysis (day 0) is around 85%.

3.6 Immuno- cytochemistry Analysis of Differentiation Markers

For the analysis of the extent of differentiation into PPS and IM, cells differentiated onto glass coverslips coated with vitronectin (*see* Subheadings 3.2 and 3.3) are collected at day -1 and day 0, respectively. For the analysis of the extent of differentiation into NPCs, RVs, and nephron structures (*see* Subheading 3.4), organoids are collected at days 5, 8, and 16, respectively. Immunocytochemistry is performed in whole-mount organoids. Alternatively, paraffin sectioning of organoids can be also performed (*see* **Note 12** for details on kidney organoid sample processing for paraffin sectioning and immunohistochemistry).

1. Remove the media from the correspondent wells containing the glass coverslips with cells. For organoids, remove the media from the transwells. Wash samples once with $1\times$ PBS.
2. In the fume hood, fix the samples by adding 1 mL of 4% paraformaldehyde to each well. For organoids on transwells, add 0.5 mL of 4% paraformaldehyde inside the transwell and 1 mL of 4% paraformaldehyde in the base of the transwell. Incubate for 20–30 min at RT in the case of cells and 1 h at RT in the case of organoids.
3. In the fume hood, remove the fixative and wash the samples three times with $1\times$ PBS at RT for 5 min each in the case of cells and three times for 15 min each in the case of organoids.
4. Block the samples with 500 μ L of $1\times$ TBS containing 1% Triton X-100 and 6% donkey serum. For organoids on transwells, add 0.5 mL of the blocking solution inside the transwell and 1 mL of the blocking solution in the base of the transwell. Incubate for 1 h at RT in the case of cells and 4 h at RT in the case of organoids. In this step and during the next steps (**steps 5–12**), place the samples over a shaker when possible.
5. For nephron structure analysis in which the biotinylated Lotus Tetragonolobus Lectin (LTL) is used to stain proximal tubule-like structures, an additional blocking step with the Streptavidin/Biotin blocking kit is required to block the organoid endogenous biotin. Briefly, use 4–5 drops of streptavidin solution and incubate for 20 min. Wash once with $1\times$ TBS for 15 min at RT in the case of cells and 15 min at RT in the case of organoids. Then use 4–5 drops of biotin solution and incubate 20 min. Wash once with $1\times$ TBS for 15 min at RT in the case of cells and 15 min at RT in the case of organoids. Notice that after using the Streptavidin/Biotin blocking kit, the solutions to dilute primary and secondary antibodies contain 1% BSA instead of donkey serum (*see* next **steps 6–10**).
6. After blocking, prepare the correspondent primary antibody combinations. At this point, the transwell membranes containing organoids are cut using a scalpel and placed into separate wells of a 24-well plate. Primary antibody combinations and

antibody dilutions are detailed below. Antibodies in combinations a., b., c., and d. are diluted in $1\times$ TBS containing 0.5% Triton X-100 and 6% donkey serum. Antibodies and LTL in combination e. are diluted in $1\times$ TBS containing 0.5% Triton X-100 and 1% BSA.

- (a) For PPS analysis (day -1): Brachyury (1:20) + Oct4 (1:25).
 - (b) For IM analysis (day 0): PAX2 (1:20).
 - (c) For NPCs analysis (day 5): SALL1 (1:100) + SIX2 (1:500).
 - (d) For renal vesicles analysis (day 8): PAX2 (1:20) + WT1 (1:100).
 - (e) For nephron structures analysis (day 16): LTL (1:200) + Podocalyxin (PODXL) (1:100) + E-cadherin (ECAD) (1:50).
7. Remove the blocking buffer and incubate the samples with primary antibodies overnight at 4 °C. Use 250- μ L volume per well of a 24-well plate.
 8. The following day, wash the samples with $1\times$ TBS containing 0.5% Triton X-100 and 6% donkey serum (instead of donkey serum, use 1% BSA for samples assayed for LTL) three times for 5 min each at RT in the case of cells and three times for 15 min each at RT in the case of organoids.
 9. Prepare the correspondent secondary antibody combinations and dilutions as detailed below. Antibodies in combinations a., b., c., and d. are diluted in $1\times$ TBS containing 0.5% Triton X-100 and 6% donkey serum. Antibodies and LTL in combination e. are diluted in $1\times$ TBS containing 0.5% Triton X-100 and 1% BSA:
 - (a) For PPS analysis (day -1): anti-goat 488 (1:100), anti-mouse Cy3 (1:100).
 - (b) For IM analysis (day 0): anti-goat 488 (1:100).
 - (c) For NPCs analysis (day 5): anti-mouse 488 (1:200), anti-rabbit Cy3 (1:100).
 - (d) For RVs analysis (day 8): anti-goat 488 (1:100), anti-rabbit Cy3 (1:100).
 - (e) For nephron structures analysis (day 16): DyLight 488 Streptavidin (1:40), anti-goat 555 (1:200), anti-mouse 647 (1:100).
 10. Incubate the samples with secondary antibodies diluted in $1\times$ TBS containing 0.5% Triton X-100 and 6% donkey serum (instead of donkey serum, use 1% BSA for samples assayed for LTL) for 2 h in the case of cells and 4 h in the case of organoids

at RT in the dark. Use 250 μ L volume per well of a 24-well plate.

11. Wash the samples three times with $1\times$ TBS for 5 min each at RT in the case of cells and three times for 15 min each at RT in the case of organoids.
12. For nuclei detection, incubate the samples with DAPI at a 1:5000 ratio in $1\times$ TBS for 30 min at RT in the case of cells and 1 h at RT in the case of organoids.
13. Remove the DAPI solution and mount the samples with coverslips using Fluoromount-G as mounting medium. For cells, deposit one or two drops of Fluoromount-G on top of a microscope slide, and then place the coverslip on top of it, with the growing cells facing the microscope slide. For transwell organoids, place first the transwell membrane with the organoid on top of the slide, add one or two drops of Fluoromount-G on top of the organoid, and then carefully place a round coverslip to cover the sample. Dry the excess of mounting medium and cover the borders with nail polish to fix the coverslips to the slides.
14. Keep the slides at 4 °C in darkness until microscopic observation. Sample fluorescence is well preserved for about 2 weeks. After this time, the intensity of the fluorescence staining could substantially decrease.
15. Acquire images on a confocal microscope.

4 Notes

1. It is essential for hPSCs not to be dissociated into single cells to prevent reduced attachment and poor survival. Therefore, it is best to first check if undifferentiated hPSCs are ready to detach by flushing a small volume of EDTA after 3 min of incubation at 37 °C. If cells do not come off, wait an extra minute before checking again and removing the EDTA. It is also important to visually check under the microscope the borders of the colonies since when hPSCs colonies are ready to be detached, the borders are shinier and refracting.
2. The plating dilution can vary among the hPSC cell line. When starting to culture hPSCs, try different passaging dilutions until finding the one that allows growth of the cells for approximately 5–6 days until reaching 80% confluency for next passage.
3. Always keep the cell suspension in the 15-mL tube in an incubator at 37 °C during the preparation of the cells for cell counting (Subheading 3.2, steps 3–7, and Subheading 3.4, steps 1 and 2).

4. The number of hPSCs collected out of a 10-cm Petri dish usually range from seven to ten million.
5. To have a 24-well plate for differentiation, a total number of 2.4 million cells are needed. Generally, cell suspensions are performed in 12 mL of Essential 8 media at a concentration of 2×10^5 cells/mL. In this manner, each 500 μ L contain the needed number of cells (10^5 cells) per well of a 24-well plate. The starting plating density of 10^5 cells was optimized for ES [4], H1 and H9 hPSCs lines. Importantly, the starting cell density should be tested in case other hPSCs lines are used.
6. As mentioned, plating density and cell colony distribution are essential for an efficient differentiation (Fig. 2a). If colonies are too small after 24 h of plating, wait additional 24 h before starting the differentiation (Fig. 2b).
7. After starting the differentiation, media changes and $1 \times$ PBS washes should be performed very gently. As cells start to differentiate, they tend to become looser and can easily detach from the plate, leaving spaces without cells that can greatly detriment differentiation efficiency and formation of monolayer on day 0.
8. On day 0, cells are very loosely attached to the plate surface, so a mild enzymatic treatment is sufficient to detach them and obtain a single-cell suspension.
9. Using the ES[4] human embryonic stem cell line, the monolayer differentiation of one 24-well plate (from day -4 to day 0) should be sufficient enough to generate one 96-well plate of kidney organoids (from day 0 to day 16). Expected cell numbers on day 0 should be approximately $2.5\text{--}3 \times 10^6$ cells per well of a 24-well.
10. To transfer the spheroid onto transwells, use a 200- μ L-wide orifice pipette tip to prevent damaging or fragmenting the spheroid. Carefully place the spheroid in the middle of the transwell membrane and remove with the 200 μ L-micropipette, the remaining medium from the membrane to leave only the spheroid. Afterwards, add 450 μ L of media through the side of the transwell into the base of the well to maintain the organoid under the air–liquid interface organotypic culture condition.
11. Once LIVE/DEAD fixable violet stain solution has been reconstituted, it should be used in the following 2 weeks.
12. For performing organoid sections, after organoid fixation (*see* Subheading 3.6, **steps 1–3**), place the organoids in a $7 \times 7 \times 5$ mm histology mold and embed the organoids in 0.8% low gelling temperature agarose. Once solidified, embed the block of agarose containing the organoids into paraffin following the classic histology procedure. Then, perform

5 μm thick sections using a microtome. Next, dewax and rehydrate organoid sections following the classic histology procedure. Then, proceed with an antigen retrieval consisting of citrate buffer (pH 6) at 95 °C for 30 min. Afterwards, continue with the blocking step and antibody incubations steps using 3% instead of 6% donkey serum in blocking, washing, and antibody solutions (*see* Subheading 3.6, steps 4–9). Use 1 mL of blocking buffer to incubate each slide. After blocking, use Dako pen to surround the tissue section on the slide to create a smaller area for antibody incubation. Create an area with one tissue section for negative control (only secondary antibody incubation). The Dako pen ink is water repelling and prevents diffusion of the antibody dilutions during incubations. Use 300 μL for primary and secondary antibody dilutions and incubation per slide, following the combinations described in the protocol (Subheading 3.6, steps 6–12). After DAPI incubation, add three or four drops of Fluoromount-G on top of the slide and carefully cover it with a coverslip. Dry the excess of mounting medium and seal the borders with nail polish.

Acknowledgments

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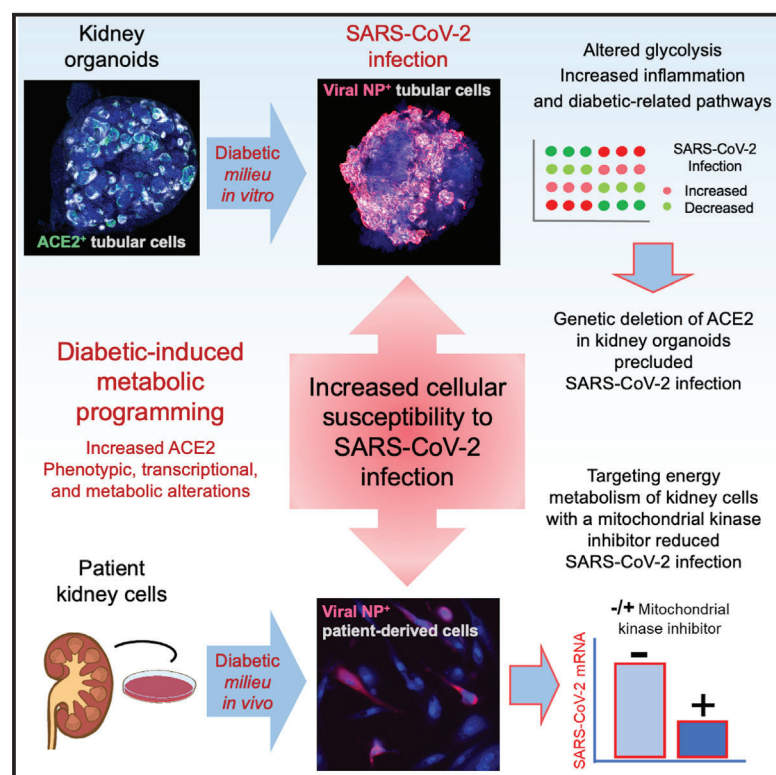
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Cell Metabolism

A diabetic milieu increases ACE2 expression and cellular susceptibility to SARS-CoV-2 infections in human kidney organoids and patient cells

Graphical abstract



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In brief

Garreta et al. developed a human kidney organoid system mirroring early hallmarks of diabetic kidney disease development. This resulted in higher SARS-CoV-2 infections, which were critically dependent on ACE2. Diabetic patient renal cells also showed elevated ACE2 and altered energy metabolism that following chemical targeting resulted in a reduction of SARS-CoV-2 infection.

Highlights

- ACE2 is upregulated in hyperglycemia in kidney organoids and patient renal cells
- Metabolic changes and increased ACE2 boost cellular infection by SARS-CoV-2
- Targeting of energy metabolism in patient renal cells lowers SARS-CoV-2 infection



Article

A diabetic milieu increases ACE2 expression and cellular susceptibility to SARS-CoV-2 infections in human kidney organoids and patient cells

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SUMMARY

It is not well understood why diabetic individuals are more prone to develop severe COVID-19. To this, we here established a human kidney organoid model promoting early hallmarks of diabetic kidney disease development. Upon SARS-CoV-2 infection, diabetic-like kidney organoids exhibited higher viral loads compared with their control counterparts. Genetic deletion of the angiotensin-converting enzyme 2 (ACE2) in kidney organoids under control or diabetic-like conditions prevented viral detection. Moreover, cells isolated from kidney biopsies from diabetic patients exhibited altered mitochondrial respiration and enhanced glycolysis, resulting in higher SARS-CoV-2 infections compared with non-diabetic cells. Conversely, the exposure of patient cells to dichloroacetate (DCA), an inhibitor of aerobic glycolysis, resulted in reduced SARS-CoV-2 infections. Our results provide insights into the identification of diabetic-induced metabolic programming in the kidney as a critical event increasing SARS-CoV-2 infection susceptibility, opening the door to the identification of new interventions in COVID-19 pathogenesis targeting energy metabolism.



INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 patients display influenza-like symptoms ranging from mild disease to severe lung injury. A high percentage of severe COVID-19 patients display symptoms in other organs, most notably the gastrointestinal tract, cardiovascular system, and the kidney. Several conditions have been linked to the risk of developing severe COVID-19, including genetic predisposition (Ellinghaus et al., 2020; van der Made et al., 2020; Zhang et al., 2020b), immune-related responses (Bastard et al., 2021; Zhang et al., 2020a), obesity, or diabetes mellitus (DM) (Lim et al., 2021). Both COVID-19 and DM are associated with acute and chronic inflammation and both disease conditions can impact each other in terms of clinical progression and disease outcome (Ugwueze et al., 2020). Notably, SARS-CoV-2 infections lead to acute kidney injury (AKI) in >20% of hospitalized patients (Nadim et al., 2020), and higher rates of mortality have been reported in patients with pre-existing DM (Khalili et al., 2021; Legrand et al., 2021).

We have previously shown that both kidney and vascular organoids derived from human pluripotent stem cells (hPSCs) support SARS-CoV-2 infections, which was blocked in the presence of clinical-grade human recombinant soluble angiotensin-converting enzyme 2 (ACE2) (Monteil et al., 2020). However, besides the great utility of organoids in SARS-CoV-2 research, organoids have not yet been developed that model human co-morbidities associated with severe COVID-19, such as DM.

Here, we show that high glucose oscillations in engineered human kidney organoids led to phenotypic, transcriptional, and metabolic alterations reminiscent to human kidney disease development in a diabetic milieu. These diabetic conditions enhanced ACE2 expression and SARS-CoV-2 infection, which was validated in human proximal tubular cells isolated from diabetic kidney biopsies. Genetic deletion of ACE2, but not BSG/CD147 or NRP1, other candidate receptors for SARS-CoV-2, completely abrogated SARS-CoV-2 infections under normal and diabetic conditions. This study provides mechanistic evidence on metabolic alterations that can increase cellular susceptibility to SARS-CoV-2 infections and unequivocally establishes ACE2 as the critical SARS-CoV-2 receptor in the human kidney, even under diabetic conditions.

RESULTS

Establishment of diabetic human kidney organoids

Diabetic patients exhibit oscillatory levels in glucose with daily episodes of hypo- and hyperglycemia, which is thought to be important for driving DM-associated pathologies (Vasquez-Muñoz et al., 2021). Indeed, the term “metabolic memory” coins the pathogenic alterations induced by hyperglycemia long after accomplishment of glycemic control (Genuth et al., 2002; Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group, 2003). To emulate *in vitro* diabetic-patient-like oscillations in glucose levels, we established a procedure

to generate diabetic-like kidney organoids from hPSCs by adapting our previous protocol (Monteil et al., 2020). We delineated a culture setup using continuous low glucose (5 mM, termed “control” conditions) or high glucose in an oscillatory fashion (alternating 5–25 mM every 24 h, termed “diabetic” conditions) (Figure 1A). Kidney organoids under both control and diabetic culture conditions showed the presence of glomerular-like and renal-tubular-like structures to a similar extent, as determined by immunodetection of PODXL⁺ podocyte-like cells and *Lotus Tetraglobus* lectin proximal tubular-like cells (LTL)⁺ (Figure S1A) and qPCR analysis for the proximal tubule marker gene *SLC3A1* and the podocyte marker genes *NPHS1*, *PODXL*, *WT1*, and *MAFB* (Figure S1B). Similarly, the mRNA expression levels of the endothelial marker genes *Endoglin*, *VGFR*, and *PDGFRα* and the stromal marker genes *vimentin* and *MEIS1/2/3* did not show significant variations between culture conditions (Figure S1B). Transmission electron microscopy (TEM) showed that tubular-like and podocyte-like cells in both control and diabetic conditions exhibited typical late-stage renal differentiation features (Figure S1C).

Changes in kidney extracellular matrix (ECM) proteins are associated with renal fibrosis and chronic kidney disease (CKD) in diabetes (Bülow and Boor, 2019; Yang et al., 2015). Importantly, high oscillatory glucose treatment resulted in increased collagen fiber deposition in kidney organoids compared with low-glucose controls (Figures 1B–1D; Data S1). We also observed an upregulation of collagen III and collagen IV mRNA expression by qPCR (Figure 1E). Similarly, fibronectin deposits were detected in the tubulointerstitial areas within kidney organoids (Figure 1F; Data S1). PAS staining and immunohistochemistry for the basement membrane proteins laminin and collagen IV in combination with the podocyte marker nephrin and proximal tubule marker LTL showed that the composition and integrity of basement membranes were largely preserved under diabetic conditions (Figures S1D and S1E; Data S1). The glycolysis-associated genes *HK2* and *LDHA* were upregulated in the diabetic kidney organoids (Figure S1F), whereas *PGC1α* (a key regulator of mitochondrial biogenesis involved in diabetic nephropathy) (Li and Susztak, 2018) was downregulated (Figure S1G).

To assess whether the diabetic milieu altered cellular metabolism, we isolated proximal tubular-like cells from kidney organoids by fluorescent-activated cell sorting of the LTL⁺ cell fraction (Figures S2A and S2B). LTL⁺ cells isolated from both experimental conditions were expanded in 5 mM glucose medium for 2 months until passage 5–7 in culture. After this time, *PGC1α* expression in LTL⁺ cells was significantly decreased in cells isolated from diabetic organoids compared with control organoids as shown by qPCR (Figure S2C) and immunofluorescence (Figure S2D). The expression of the tubular markers LTL and Na-K ATPase was unchanged (Figure S2D). The reduction in *PGC1α* levels was concomitant with increases in the maximum oxygen consumption rate (OCR), a measurement of mitochondrial respiration, basal and maximal respiration, as well ATP synthesis (Figure 1G). Of note, our results are in line with other studies at early phases of diabetes in animal models (1–4 weeks after induction of diabetes) (Friederich et al., 2008; Friederich-Persson and Persson, 2020). In summary, our data show that exposure

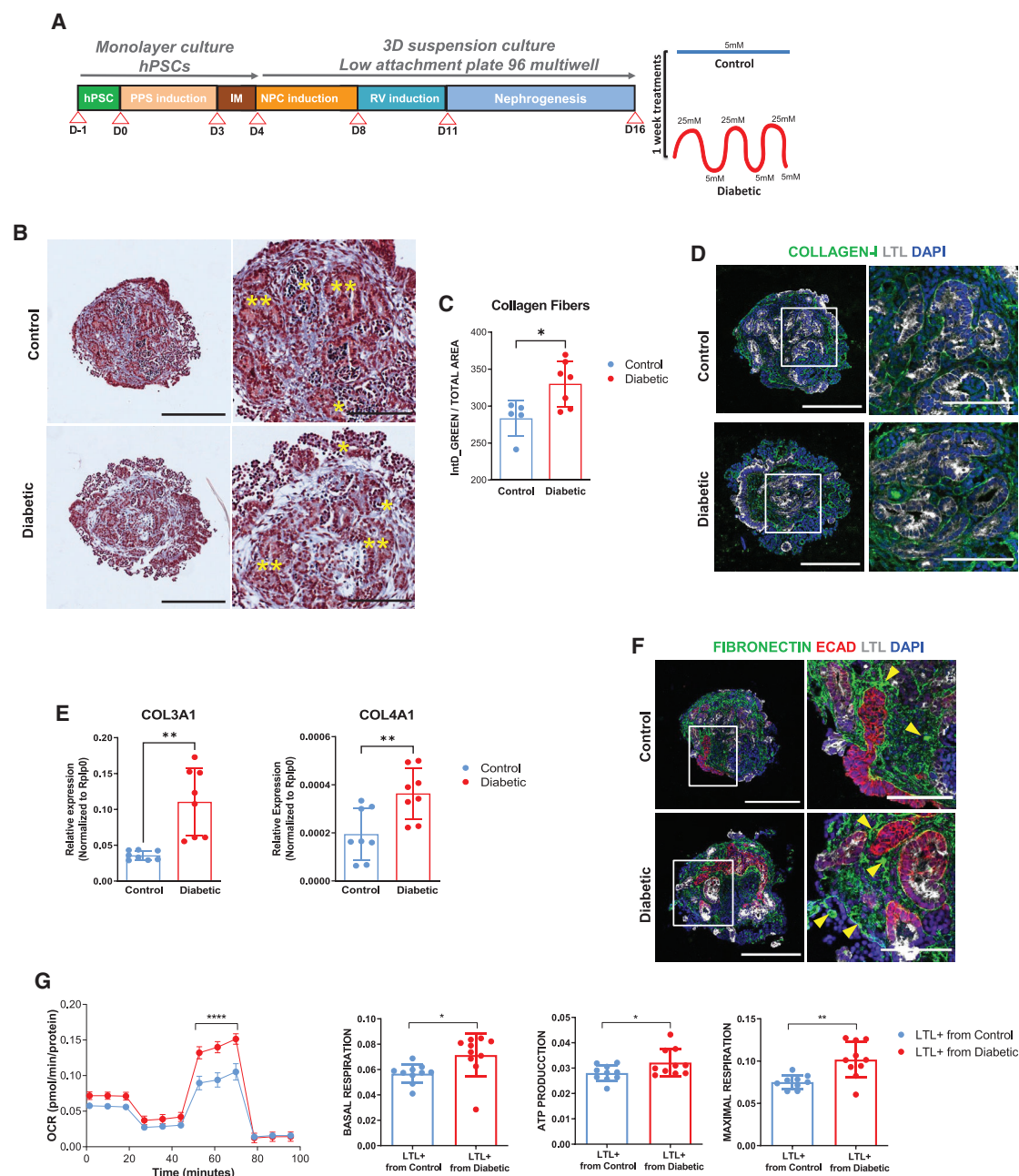


Figure 1. High oscillatory glucose conditions induce early hallmarks of the diabetic kidney disease in human kidney organoids

(A) Experimental scheme for the generation of human kidney organoids from hPSCs.

(B) Trichrome Masson staining of control or diabetic kidney organoids. Glomerular (*) and tubular (**) structures are shown. Scale bars, 250 and 100 μ m (magnified views).

(C) Corresponding quantification of collagen fibers (B). y axis represents integrated intensity. Data are mean $\pm$ SD of at least n = 5 independent experimental replicates per condition. *p < 0.05, unpaired Student's t test.

(D) Representative immunofluorescence staining for COLLAGEN-I (green), LTL (gray), and DAPI (blue) in control or diabetic kidney organoids. Scale bars, 250 and 100 μ m (magnified views).

(E) mRNA expression levels of COL3A1 and COL4A1 in control or diabetic kidney organoids. Data are mean $\pm$ SD. n = 3 independent biological replicates from a pool of 12 organoids/group with two technical replicates each. **p < 0.01, unpaired Student's t test.

(F) Representative immunofluorescence staining for FIBRONECTIN (green), E-CADHERIN (ECAD; red), LTL (gray), and DAPI (blue) in control or diabetic kidney organoids. Scale bars, 250 and 100 μ m (magnified views). Yellow arrows highlight sites of fibronectin deposits.

(G) Seahorse analysis in LTL⁺ cells isolated from control or diabetic kidney organoids. The oxygen consumption rate (OCR) data are normalized to total protein. Data are mean $\pm$ SD. n = 10 biological replicates/group. ****p < 0.0001, two-way ANOVA, followed by Bonferroni post-test. Basal respiration and spare respiratory capacity, cellular ATP production, and maximal respiration are shown as mean $\pm$ SD. n = 10 biological replicates/group. *p < 0.05; **p < 0.005, unpaired Student's t test.

See also Figures S1 and S2 and Data S1.

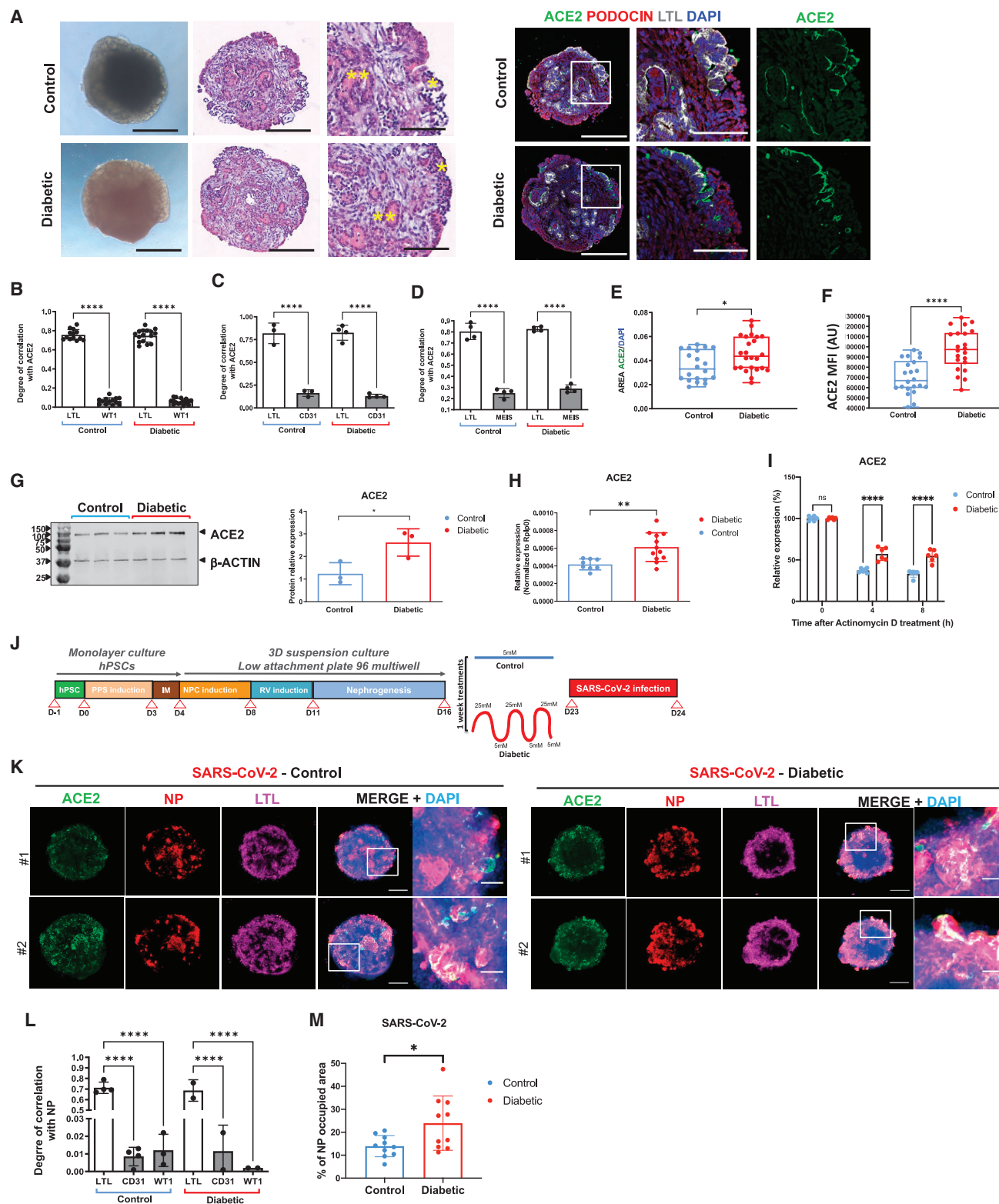


Figure 2. Diabetic conditions induce ACE2 expression in human kidney organoids and enhance SARS-CoV-2 infections

(A) Representative bright-field images and hematoxylin and eosin staining of control or diabetic kidney organoids. Asterisks highlight podocyte-like cells (*) or tubular-like structures (**). Consecutive sections were stained for ACE2 (green), PODOCIN (red), and using *Lotus Tetraglobus* lectin (LTL, tubular cell marker, gray) and DAPI (blue). Scale bars, 250 and 100 μ m (magnified views).

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of human kidney organoids to high oscillatory glucose leads to transcriptional changes, ECM alterations, and metabolic mitochondrial rewiring in tubular cells, early hallmarks of kidney disease development induced by hyperglycemia. Our culture system also allowed for the generation of proximal tubule-like cells (LTL⁺) recapitulating hallmarks of an early diabetogenic-like phenotype after removal of the diabetogenic insult, indicative of metabolic memory.

Diabetic conditions induce ACE2 expression in human kidney organoids

ACE2 has been previously identified as a key host cell surface receptor sufficient for SARS-CoV-2 entry into host cells (Walls et al., 2020; Wan et al., 2020; Wrapp et al., 2020). Of note, ACE2 has also been previously shown to control the progression of CKD in multiple animal models (Maksimowski et al., 2020). Although it has been reported that glucose can induce ACE2 expression in cell lines (Härdtner et al., 2013), it is still controversial whether DM results in up- or downregulation of ACE2 (Mizuri et al., 2008; Reich et al., 2008; Sandooja et al., 2020), making it paramount to test the effects of glucose on ACE2 expression in complex human tissue-like cultures as organoids. Our analysis indicated that in the kidney organoids, ACE2-expressing cells (ACE2⁺) are predominantly detected in LTL⁺ (Figure 2A; Data S1). Image quantification of ACE2⁺LTL⁺ was evaluated together with markers of different renal compartments including the podocyte marker WT1, the endothelial cell marker CD31, and the stroma marker MEIS1/2/3 (Figures 2B–2D; Data S1), overall showing a similar expression pattern of ACE2 to that found in the native human and mouse kidney (Deng et al., 2020; Lin et al., 2021). Importantly, the oscillatory glucose treatment promoted a significant upregulation of ACE2 expression compared with control conditions at the protein (Figures 2E–2G and S2E–S2G) and mRNA (Figure 2H) levels. Since mRNA stability plays a major role in gene expression regulation, we hypothesized that hyperglycemia might affect the

half-life of ACE2 mRNA. To test this idea, kidney organoids were challenged with the RNA transcription inhibitor actinomycin D leading to an increase of ACE2 mRNA stability in diabetic organoids compared with control counterparts (Figure 2I).

Increased SARS-CoV-2 infection in diabetic kidney organoids

Next, control and diabetic kidney organoids were infected with SARS-CoV-2, recovered, and analyzed at 1 day post-infection (1 dpi) (Figure 2J). Control organoids were productively infected as detected by immunostaining for the viral nuclear protein (NP) (Figure 2K). Infected cells within organoids were primarily LTL⁺ (Figure 2L; Data S1). Remarkably, diabetic organoids showed significantly enhanced SARS-CoV-2 infections compared with control organoids, as quantified by the percentage of cells positive for NP expression (NP⁺) by confocal microscopy (Figure 2M; Data S1). To assess potential transcriptional changes induced by SARS-CoV-2, single-cell RNA sequencing (scRNA-seq) was performed across 4 biological conditions (mock and 1 dpi, in both 5 mM glucose- and high oscillatory glucose-treated kidney organoids). Cell types in kidney organoids were assigned using unsupervised clustering after integrating control versus diabetic infected conditions and the uniform manifold approximation and projection (UMAP) algorithm to visualize the scRNA-seq data (Figure 3A). We retrieved renal endothelial-like, mesenchymal, proliferating, podocyte, and tubule cell populations in all four experimental samples, indicating that apparently neither diabetic conditions nor SARS-CoV-2 infections altered cell compositions in the kidney organoids (Figure S3A). Organoids from the all four conditions contained cells representative of a developing nephron, including ENG⁺ and PECAM1⁺ (endothelial-like cells), NPHS1⁺ and NPHS2⁺ (podocyte-like cells), LRP2⁺ and SLC3A1⁺ (tubule proximal-like cells), and CDH1⁺ (distal-like tubular cells) (Figure S3B), also resembling second trimester human fetal kidney cell populations (Figure S3C). Diabetic kidney

(B) Quantification of ACE2 colocalization with LTL⁺ and WT1⁺ cells in control or diabetic kidney organoids. Data are mean ± SD. n = 13 (control) and n = 16 (diabetic) organoids per condition. ****p < 0.0001, one-way ANOVA, Tukey's multiple comparisons test.

(C) Quantification of ACE2 colocalization with LTL⁺ and CD31⁺ cells in control or diabetic kidney organoids. Data are mean ± SD. n = 3 (control) and n = 4 (diabetic) organoids per condition. ****p < 0.0001, one-way ANOVA, Tukey's multiple comparisons test.

(D) Quantification of ACE2 colocalization with LTL⁺ and MEIS⁺ cells in control or diabetic kidney organoids. Data are mean ± SD. n = 4 (control) and n = 4 (diabetic) organoids per condition. ****p < 0.0001, one-way ANOVA, Tukey's multiple comparisons test.

(E) Quantification of the area of ACE2⁺ cells in control or diabetic kidney organoids. Data are mean ± SD. n = 20 (control) and n = 24 (diabetic) organoids. *p < 0.05, unpaired Student's t test.

(F) Quantification of the mean fluorescence intensity (MFI as arbitrary units [AU]) of ACE2⁺ cells in control or diabetic kidney organoids. Data are mean ± SD. n = 6 (control) and n = 6 (diabetic) organoids. ****p < 0.0001, unpaired Student's t test.

(G) Protein levels of ACE2 in control or diabetic kidney organoids are shown by western blot. β-actin was used as loading control. The correspondent quantification is shown. Data are mean ± SD. n = 3 independent biological replicates from a pool of 12 organoids/group; *p < 0.05, unpaired Student's t test.

(H) mRNA expression levels of ACE2 in control or diabetic kidney organoids. Data are mean ± SD. n = 4 independent experimental replicates from a pool of 12 organoids/group with two technical replicates each. **p < 0.005, unpaired Student's t test.

(I) mRNA expression levels of ACE2 in control or diabetic kidney organoids upon actinomycin D treatment. Data are mean ± SD. n = 2 independent biological replicates from a pool of 12 organoids/group with three technical replicates each. ns, no statistical significance; ****p < 0.0001, two-way ANOVA followed by Bonferroni post-test.

(J) Experimental scheme for the infection of control or diabetic kidney organoids with SARS-CoV-2.

(K) Immunofluorescence of control and diabetic kidney organoids at 1 dpi with SARS-CoV-2 for ACE2 (green), viral nuclear protein (NP, red), LTL (magenta), and DAPI (blue). Scale bars, 250 and 50 μm (magnified views). n = 2 organoids per condition.

(L) Quantification of viral NP colocalization with LTL⁺, CD31⁺, and WT1⁺ cells in control or diabetic kidney organoids. Data are mean ± SD. n = 3 control and n = 3 diabetic organoids. ****p < 0.0001, one-way ANOVA, Tukey's multiple comparisons test.

(M) Quantification of the area of NP⁺ cells from images in (K). Data are mean ± SD. n = 5 control and n = 5 diabetic kidney organoids performing two technical replicates each. *p < 0.05, unpaired Student's t test.

See also Figure S2 and Data S1.



(B) UMAPs for SARS-CoV-2 expression in control or diabetic kidney organoids at 1 dpi. For SARS-CoV-2, expression is considered as undetectable for cells expressing <5 unique molecular identifiers (UMIs). Cells are colored based on expression level. The violin plots in the bottom panels represent ≥ 5 UMI expression levels for the different cell types indicated.

(D) Differentially expressed genes (DEGs) in the comparison of the SARS-CoV-2-infected against mock organoids in diabetic conditions considering only renal-like cell types. In the volcano plot, the x axis indicates log fold change (FC), and the y axis indicates statistical significance with the $-\log_{10}(\text{p value})$. Genes with an adjusted p value < 0.05 are considered upregulated (red) if the $\log\text{FC} > 0.1$ and downregulated (blue) if the $\log\text{FC} < -0.1$. Non-DEGs are shown in gray.

See also [Figures S3](#) and [S4](#).

organoids at 1 dpi again showed increased numbers of cells containing viral RNA compared with control organoids (Figure 3B). As expected from an active infection, SARS-CoV-2 infections resulted in an increase of inflammatory-related processes in kidney organoids under control and diabetic conditions (Figure 3C) in agreement with previous findings in kidney organoids showing increased profibrotic signaling, cellular

injury, and inflammatory responses driven by SARS-CoV-2 infection (Jansen et al., 2021). Our results also showed that SARS-CoV-2 infections were also associated with downregulation of glycolysis-related processes in diabetic organoids (Figure 3C). In addition to altered glycolysis, differential gene expression analysis showed increased inflammation (e.g., *CXCL* family genes) and diabetic-related pathways (e.g., *CEBPD* and *STAT3*)

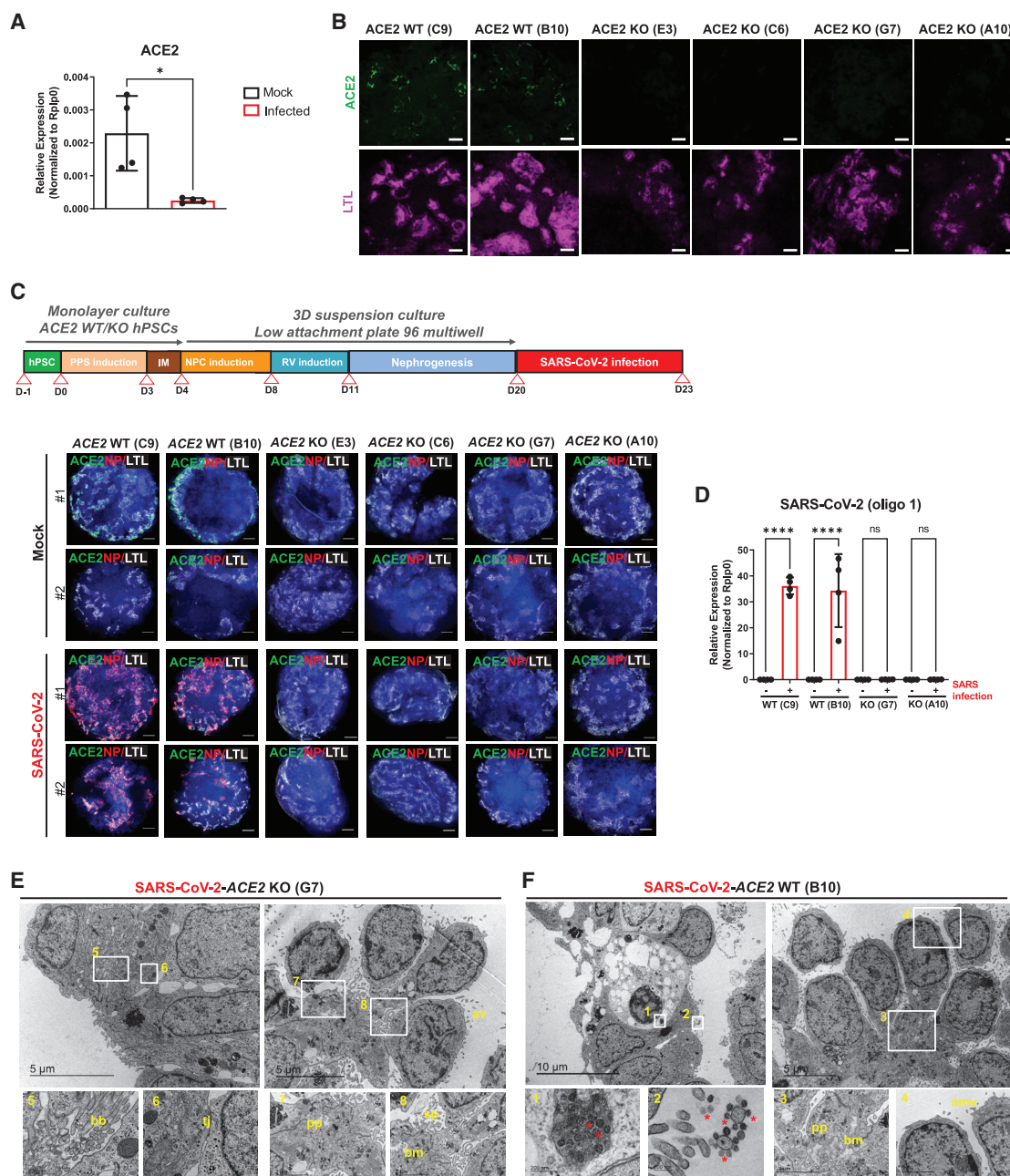


Figure 4. SARS-CoV-2 infection in human kidney organoids depends on ACE2

(A) mRNA expression levels of *ACE2* in mock or SARS-CoV-2-infected kidney organoids. Data are mean $\pm$ SD. $n = 2$ independent biological replicates from a pool of 12 organoids/group with two technical replicates each. * $p < 0.05$, unpaired Student's t test.

(B) Immunofluorescence staining for *ACE2* (green) and LTL (magenta) in WT and *ACE2* KO kidney organoids. Scale bars, 50 μ m.

(C) Experimental scheme for the infection with SARS-CoV-2 of WT and *ACE2* KO kidney organoids. Immunofluorescence of mock or SARS-CoV-2-infected WT and *ACE2* KO kidney organoids at 3 dpi for *ACE2* (green), viral nuclear protein (NP, red), LTL (gray), and DAPI (blue). Scale bars, 250 μ m.

(D) mRNA expression of SARS-CoV-2 and *ACE2* in mock or SARS-CoV-2-infected WT and *ACE2* KO kidney organoids at 3 dpi by qPCR. Data are mean $\pm$ SD. $n = 2$ independent biological replicates from a pool of 12 organoids/group with two technical replicates each. **** $p < 0.0001$; ns, no statistical significance; one-way ANOVA, Tukey's multiple comparisons test.

(E) TEM analysis of WT and *ACE2* KO kidney organoids infected with SARS-CoV-2 and recovered at 3 dpi. Representative images of infected *ACE2* WT specimen show numerous viral particles (asterisks) inside a vesicle near the plasma membrane of a dying cell (1) and in the apical microvilli (amv) of a tubular-like cell (2). Details for podocyte-like cells exhibiting podocyte-related structures including primary processes (pp) (3), the deposition of a basement membrane (bm) (3),

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in SARS-CoV-2-infected diabetic kidney organoids compared with non-diabetic counterparts (Figures 3D and S3D–S3F).

Recent findings showed that glycolysis sustains SARS-CoV-2 replication in human monocytes exposed to high glucose (11 mM) (Codo et al., 2020). Considering our findings that SARS-CoV-2 infection in diabetic kidney organoids led to a decrease in glycolysis, we also investigated the impact of SARS-CoV-2 infection in kidney organoids exposed to 11 mM glucose culture conditions. SARS-CoV-2 infections did not alter cell clustering or cell-type proportions across mock and 1 dpi kidney organoids grown in 11 mM glucose (Figures S4A and S4B). Moreover, cells presenting high expression of viral RNA were mainly located in the loop of Henle and proximal tubule cell clusters, again co-expressing ACE2 (Figure S4C). Importantly, following SARS-CoV-2 infections, we observed a switch from oxidative phosphorylation (OXPHOS) to a glycolytic-based metabolism in the kidney organoids exposed to 11 mM glucose in agreement with recent findings in human monocytes (Figure S4D) (Codo et al., 2020). Analysis for differentially expressed genes confirmed those findings together with enrichment in IFN and TNF, IL-2/6, and mTORC1 signaling (Figures S4D–S4F).

ACE2 is essential for SARS-CoV-2 infections in kidney organoids

The ACE2 receptor is sufficient for SARS-CoV-2 entry into human cells, though other receptors have been proposed (Hoffmann et al., 2020). Whether ACE2 is essential for SARS-CoV-2 infections is still not known. We and others have previously shown that ACE2 expression protects the lung from injury and that its expression is downregulated *in vivo* and *in vitro* upon SARS-CoV and SARS-CoV-2 spike protein engagement on the cell membrane (Haga et al., 2008; Imai et al., 2005; Kuba et al., 2005; Triana et al., 2021). Here, we found that ACE2 mRNA levels were significantly downregulated in kidney organoids after SARS-CoV-2 infection compared with mock conditions (Figure 4A).

To test whether ACE2 is indeed essential for SARS-CoV-2 infections in kidney organoids, we generated ACE2 knockout (KO) hPSC lines using CRISPR-Cas9 genome editing (Figures S5A and S5B). Upon differentiation, H&E staining showed that both wild-type (WT) and ACE2 KO kidney organoids exhibited similar nephron-like structures containing podocyte-like and tubule proximal-like cells (Figure S5C). ACE2 deficiency was confirmed by immunofluorescence (Figure 4B) and western blotting (Figure S5D). qPCR analysis of WT and ACE2 KO kidney organoids showed the detection of prototypic renal markers (Figure S5E) with no apparent changes in renal cell populations as revealed by scRNA-seq (Figures S6A and S6B). In addition, the expression of basigin (BSG, also known as CD147), proposed as another putative receptor for SARS-CoV-2 (Wang et al., 2020), was not affected by ACE2 deficiency as shown by immunofluorescence (Figure S6C). Interestingly, ACE2 KO kidney organoids exhibited an upregulation in OXPHOS processes compared with

WT organoids as shown by gene set enrichment analysis (GSEA) (Figure S6D). These results are in line with previous observations that genetic deletion of ACE2 or pharmacological inhibition of ACE2 induces renal oxidative stress and promotes diabetic renal injury (Soler et al., 2007; Wysocki et al., 2014).

Next, control WT and ACE2 KO kidney organoids were infected with SARS-CoV-2 and virus infection was monitored at 3 dpi (Figure 4C). Downregulation of ACE2 expression upon infection in WT organoids was re-confirmed at the protein level (Figure S6E). SARS-CoV-2 infection in ACE2 WT organoids was shown based in viral NP detection in LTL⁺ cells by immunofluorescence (Figure 4C) and the expression of SARS-CoV-2 mRNA by qPCR (Figures 4D and S7). In contrast, ACE2 deletion in ACE2 KO kidney organoids resulted in a complete absence of NP⁺ cells and almost undetectable levels of SARS-CoV-2 mRNA (Figures 4C, 4D, and S7). TEM analysis corroborated the absence of SARS-CoV-2 viral particles in ACE2 KO kidney organoids (Figure 4E), whereas viral particles were present in the WT background (Figure 4F). No significant changes in the levels of expression of podocyte marker genes (*WT1*, *PODXL*, *NPHS1*, *NPHS2*, and *MAFB*) or for tubular marker genes (*SLC3A1* and *SLC16A1*) were found after infection comparing WT and ACE2 KO kidney organoids (Figure S7).

Impact of SARS-CoV-2 infections in BSG and NRP1 KO kidney organoids

To further validate the unique role of ACE2 in SARS-CoV-2 infection in kidney organoids, we generated BSG KO hPSCs using CRISPR-Cas9 (Figures 5A and 5B). Upon differentiation, both the respective WT and BSG KO kidney organoids exhibited the presence of nephron-like structures containing podocyte-like cells and tubular structures (Figure 5C). BSG deletion in BSG KO kidney organoids was confirmed using qPCR (Figure 5D). We also generated NRP1 KO hPSCs (Figures S8A and S8B), confirming NRP1 deletion in NRP1 KO kidney organoids by western blotting (Figure S8C). To determine if BSG or NRP1 also plays a role in SARS-CoV-2 infection, as previously proposed (Cantuti-Castelvetri et al., 2020; Daly et al., 2020; Wang et al., 2020), WT and KO kidney organoids for BSG and NRP1 were infected with SARS-CoV-2 and analyzed at 3 dpi (Figures 5E and S8D, respectively). TEM analysis revealed the presence of viral particles in both WT and BSG KO organoids (Figure 5F). These results were confirmed by immunofluorescence for the detection of NP⁺ (Figure 5G). Interestingly, we found decreased levels of SARS-CoV-2 mRNA expression in the infected BSG KO kidney organoids compared with WT counterparts (Figure 5H), suggesting that BSG deletion might have an impact on the infection susceptibility of the organoids. In contrast, the presence of NP⁺ and SARS-CoV-2 mRNA expression levels were comparable between WT and NRP1 KO kidney organoids upon infection (Figures S8E and S8F). Overall, these findings do not support an indispensable role for BSG or NRP1 in SARS-CoV-2 infections in human kidney organoids.

and apical microvilli (amv) (4) are shown. Scale bars, 10 and 5 μ m, 200 nm (magnified views in 1 and 2), and 2 μ m (magnified views in 3 and 4). Representative images of infected ACE2 KO specimen show epithelial tubular-like cells with brush borders (bb) (5) and tight junctions (tj) (6). Details for podocyte-like cells with podocyte-related structures including primary (pp), the deposition of a basement membrane (bm), and cell processes (sp) are shown. Scale bars, 5 μ m, 500 nm (magnified views in 5 and 6), and 2 μ m (magnified views in 7 and 8). See also Figures S5–S7.

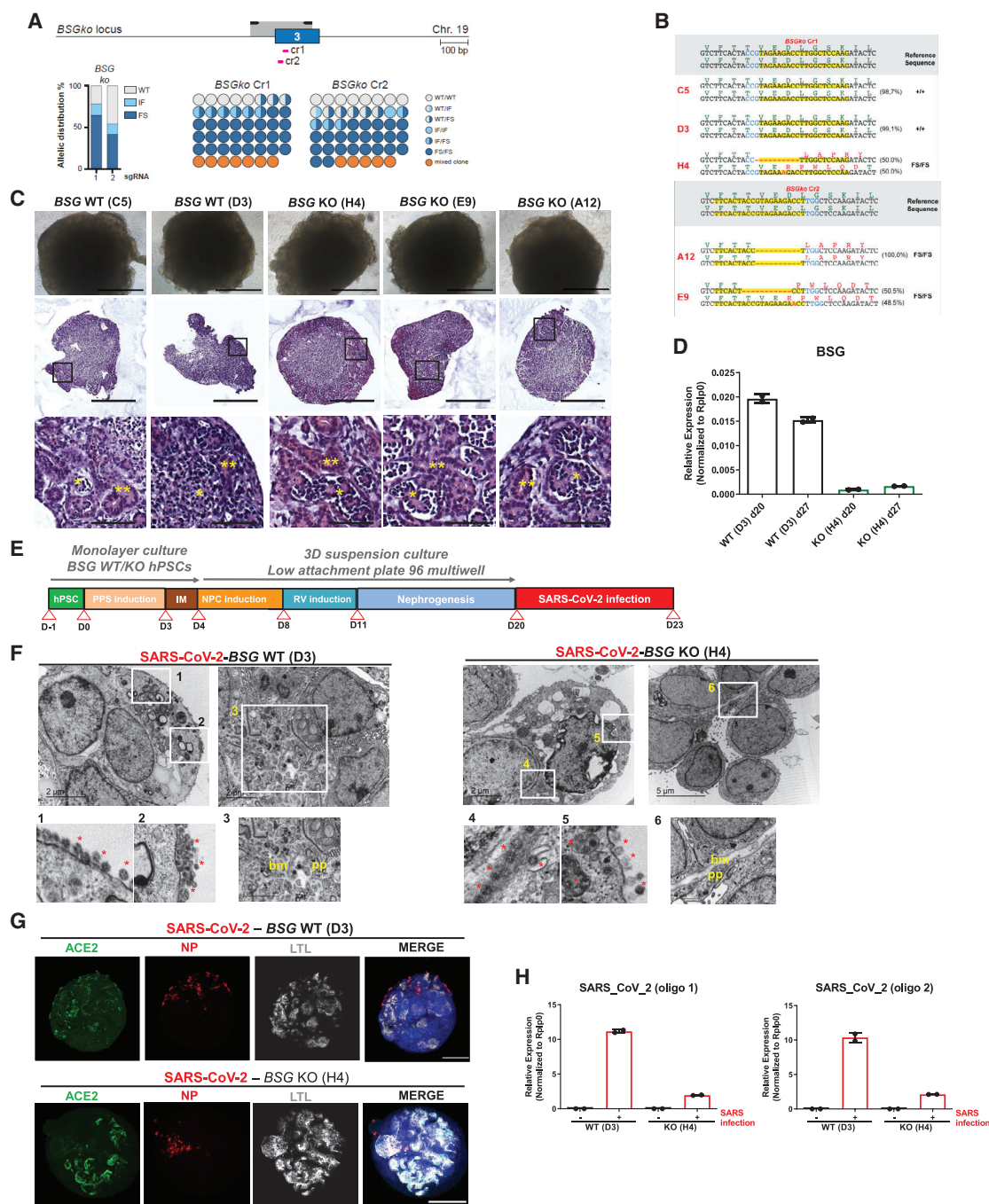


Figure 5. SARS-CoV-2 infections in BSG KO kidney organoids

(A) Schematic of Cas9/gRNA-targeting sites (pink arrows) in *BSG* locus showing exon structure (blue boxes) and PCR amplicons (light gray boxes in all figures in this study). Histogram shows allelic sequence distribution after the transfection of the different gRNAs in undifferentiated ES[4] cells expressing an inducible Cas9 (iCas9). WT, wild type; mut, mutation; FS, frameshift.

(B) Representative sequence of the wild type (+/+) or BSG mutant clones generated with the different gRNAs.

(C) Representative bright-field images of WT and BSG KO kidney organoids. Scale bars, 250 μ m. Hematoxylin and eosin staining show tubular-like (**) and glomerular-like (*) structures. Scale bars, 250 and 50 μ m (magnified views).

(D) mRNA expression levels of *BSG* in WT and BSG KO kidney organoids by qPCR. Data are mean $\pm$ SD. n = 1 independent experiment from a pool of 12 organoids/group with at least two technical replicates each.

(E) Experimental scheme for the infection with SARS-CoV-2 of WT and BSG KO kidney organoids.

(F) TEM analysis of WT and BSG KO kidney organoids infected with SARS-CoV-2 at 3 dpi. Representative images of infected BSG WT specimens (left) show numerous viral particles in the cell surface of a dying cell (1 and 2). Details for podocyte-like cells (3) exhibiting podocyte-related structures including primary (pp)

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ACE2 is essential for SARS-CoV-2 infections in diabetic kidney organoids

We next assessed whether ACE2 is essential for the observed increase in SARS-CoV-2 infections under our diabetic culture conditions. To address this question, WT and ACE2 KO kidney organoids were exposed to control and diabetic conditions and analyzed at 1 dpi (Figure 6A). Importantly, infected ACE2 KO kidney organoids were negative for NP detection irrespective of the control or diabetic treatment. On the contrary, ACE2 WT kidney organoids again displayed significantly enhanced viral NP expression within the LTL⁺ structures under high oscillatory glucose conditions (Figure 6B) as confirmed by qPCR analysis (Figure 6C). Similarly, TEM analysis also confirmed the presence of viral particles in ACE2 WT kidney organoids compared with ACE2 KO counterparts (Data S1). As expected, ACE2 mRNA and protein levels remained undetectable in the diabetic ACE2 KO kidney organoids (Figures 6D and 6E). To further explore the role of ACE2 in SARS-CoV-2 infections, we tested whether restoration of ACE2 expression in the ACE2 KO kidney organoids through lentiviral transduction was sufficient to rescue SARS-CoV-2 infectivity in control and diabetic culture conditions. ACE2 transduced (ACE2t) organoids were subsequently infected with SARS-CoV-2 (Figure 6F). At 1 dpi, ACE2t kidney organoids showed similar levels of ACE2 expression (Figures 6F and 6G), and we were able to detect NP⁺ (Figure 6F). Quantification of NP⁺ in confocal images revealed that organoids exposed to diabetic conditions showed a significant increase of NP⁺ compared with their control counterparts (Figure 6H). Thus, diabetic conditions sustain enhanced SARS-CoV-2 infections in kidney organoids, which is critically dependent on ACE2 expression.

Enhanced SARS-CoV-2 infection in kidney cells from diabetic patients

Although several studies could not detect direct SARS-CoV-2 infection in kidneys (Golmai et al., 2020; el Jamal et al., 2021; Santoriello et al., 2020; Volbeda et al., 2021), other recent investigations have clearly shown that SARS-CoV-2 can directly infect renal cells (Braun et al., 2020; Diao et al., 2021; Jansen et al., 2021; Puelles et al., 2020). However, whether the kidney damage observed results from direct infection of target renal cells or indirect injury responses is not yet fully understood (Braun et al., 2020; el Jamal et al., 2021; Puelles et al., 2020; Volbeda et al., 2021). We therefore isolated kidney human proximal tubular cells (HPTCs) from kidney biopsies of non-diabetic (control) and diabetic patients (Figures 7A and S9A). The mRNA levels of *PGC1 α* were significantly downregulated in diabetic HPTCs compared with control counterparts (Figure S9B), paralleling our findings in diabetic kidney organoids and in dia-

betic kidney organoid-isolated LTL⁺ (Figures S1G and S2C). In addition, diabetic HPTCs exhibited higher OCR, basal respiration, ATP production, and maximal respiratory capacity compared with control counterparts (Figure 7B), as well as increased *LDHA* mRNA levels (Figure S9C). Of note, diabetic HPTCs showed higher ACE2 expression levels compared with control HPTCs (Figures S9D and S9E). Upon SARS-CoV-2 infection, we detected increased numbers of NP⁺ cells (Figures 7C and 7D) and elevated SARS-CoV-2 mRNA expression levels in diabetic HPTCs compared with non-diabetic controls (Figure 7E).

The diabetic-induced metabolic programming observed in HPTCs from diabetic patients prompted us to explore the molecular mechanisms at the interface between glycolysis and OXPHOS during SARS-CoV-2 infections. To assess the role of OXPHOS in viral infection, we treated the cells with DCA, an inhibitor of mitochondrial pyruvate dehydrogenase kinase (PDK) (Figure S9F), which results in activation of mitochondrial OXPHOS at the expense of glycolysis (Bonnet et al., 2007; Cook et al., 2021; Eleftheriadis et al., 2013). Importantly, when diabetic and non-diabetic patient-derived HPTCs were treated with DCA, we observed a significant decrease in SARS-CoV-2 mRNA expression levels (Figures 7F and 7G). Therefore, our results show that both altered energy metabolism and increased ACE2 impact SARS-CoV-2 infections in kidney patient cells.

DISCUSSION

There is an urgent need to better understand the complex relationships between pre-existing conditions in COVID-19 patients, which may exacerbate viral infection and disease outcomes. Diabetes has emerged as one of the most frequent co-morbidities associated with severity and mortality of COVID-19. Here, we developed a unique culture system to generate diabetic human kidney organoids, based on oscillation of glucose levels also observed in DM patients. Our approach preserved renal cell types while recapitulating early hallmarks of diabetic kidney disease (Sas et al., 2016).

When analyzing kidney organoids for the expression of the SARS-CoV-2 entry receptor ACE2, we consistently observed ACE2 positivity in tubular-like cells by confocal microscopy, confirming our previous observations using single-cell profiling in human kidney organoids (Monteil et al., 2020) and earlier reports using single-cell profiling (Zou et al., 2020) and immunohistochemistry in the mouse (Soler et al., 2013; Ye et al., 2006) and human kidney (Lely et al., 2004). Recently, single-cell profiling from 436 patients suggested that increases in ACE2 expression within lungs and kidneys may increase the risk of SARS-CoV-2 infections (Jiang et al., 2020). Our results now show that a high

and the deposition of a basement membrane (bm). Scale bars, 2 μ m, 200 nm (magnified views in 1 and 2), and 2 μ m (magnified views in 3). Representative images of infected BSG KO specimens (right) show numerous viral particles in the intercellular space (4) and in the cell surface (5). Details for podocyte-like cells (6) exhibiting podocyte-related structures including primary (pp) and the deposition of a basement membrane (bm). Scale bars, 2 and 5 μ m, 200 nm (magnified views in 4 and 5), and 1 μ m (magnified view in 6).

(G) SARS-CoV-2 mRNA expression levels in mock-treated or SARS-CoV-2-infected WT and BSG KO kidney organoids at 3 dpi by qPCR. Data are mean $\pm$ SD. n = 1 independent experiment from a pool of 12 organoids/group with at least two technical replicates each.

(H) Immunofluorescence of mock or SARS-CoV-2-infected WT and BSG KO kidney organoids at 3 dpi for ACE2 (green), viral nuclear protein (NP, red), LTL (gray), and DAPI (blue). Scale bars, 250 μ m.

See also Figure S8.

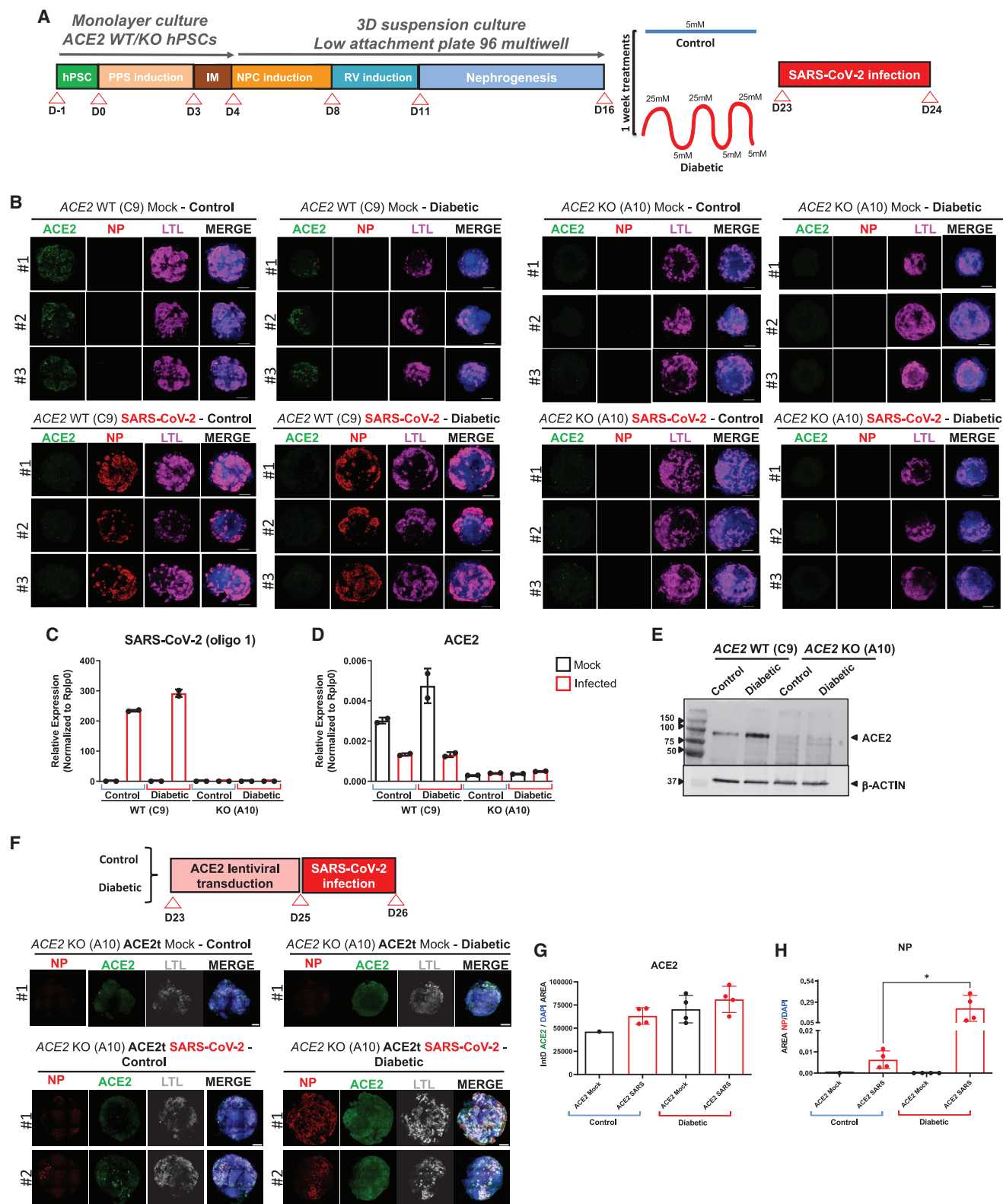


Figure 6. ACE2 expression in diabetic human kidney organoids sustains SARS-CoV-2 infections

(A) Experimental scheme for the infection with SARS-CoV-2 of WT and ACE2 KO kidney organoids under control or diabetic conditions. (B) Immunofluorescence of mock or SARS-CoV-2-infected specimens under control or diabetic conditions at 1 dpi for ACE2 (green), virus nuclear protein (NP, red), LTL (magenta), and DAPI (blue). Scale bars, 250 μ m. n = 3 organoids per condition.

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oscillatory glucose regime induces the expression of ACE2 at both the mRNA and protein levels, consistent with previous findings showing increased ACE2 levels in the kidney cortex from db/db mice and STZ diabetic mice (Ye et al., 2004) and proximal tubular cells in kidney biopsies from patients with diabetic kidney disease (Menon et al., 2020). Importantly, concurrent with enhanced ACE2 expression, we observed increased viral loads at the mRNA and protein levels in the diabetic kidney organoids. Following infection, we detected a marked decrease in ACE2 mRNA expression in accordance with previous results using colon- and ileum-derived human intestinal organoids (Triana et al., 2021). Single-cell profiling showed a decrease in OXPHOS and decrease in glycolytic-based metabolism as well as a hypoxic signature in response to SARS-CoV-2 infection in diabetic organoids.

Nearly two decades ago, we and others showed in *ace2* mutant mice that ACE2 is a negative regulator of the RAS system and genetically controls cardiovascular function and damage of multiple organs such as the lung, liver, and kidney (Clarke and Turner, 2012; Crackower et al., 2002). From the beginning of the pandemic, ACE2 took center stage in the COVID-19 outbreak as a receptor for the spike glycoprotein of SARS-CoV-2 (Wrapp et al., 2020; Zhou et al., 2020), which spurred the development of vaccines and therapies targeting the ACE2-SARS-CoV-2 spike interaction. Since various other candidate receptors have been reported (and considering the drug and vaccine development landscape), it is therefore paramount to establish whether ACE2 is not only sufficient for infection but is in fact essential. To evaluate the key role of ACE2 in SARS-CoV-2 infection, we therefore generated ACE2 KO hPSCs derived kidney organoids, which showed preserved renal differentiation; though, in line with the previously reported functions of ACE2, these ACE2 mutant organoids displayed differences with regard to OXPHOS, lipid metabolism, as well as angiogenesis/endothelium (Peña-Silva et al., 2012). To further demonstrate the unique role of ACE2 for viral entry, we also generated BSG/CD147 KO and NRP1 KO hPSC lines as others have highlighted its role in SARS-CoV-2 infection (Cantuti-Castelvetri et al., 2020; Daly et al., 2020; Shilts et al., 2021; Wang et al., 2020). However, BSG KO and NRP1 KO kidney organoids clearly supported viral infection as demonstrated by confocal microscopy and TEM analysis, thus excluding an essential role of BSG or NRP1 in renal SARS-CoV-2 infections.

For many of the systemic manifestations of COVID-19, it is unclear whether the pathology is a secondary “side effect” of the

SARS-CoV-2 infection, such as immune activation or altered coagulation, or whether it is also due to a direct SARS-CoV-2 infection of specific organs. We first described that kidney organoids can shed progeny SARS-CoV-2 viruses (Monteil et al., 2020, 2021), and later investigations confirmed SARS-CoV-2 kidney tropism (Braun et al., 2020; Diao et al., 2021; Jansen et al., 2021; Puelles et al., 2020), including the ability to replicate in human kidney cells, thereby establishing an association of kidney infection by SARS-CoV-2 with shorter survival time and increased incidence of AKI in COVID-19 patients (Braun et al., 2020; Puelles et al., 2020). However, it should be noted that other studies failed to detect SARS-CoV-2 in kidneys (Golmai et al., 2020; el Jamal et al., 2021; Santoriello et al., 2020; Volbeda et al., 2021). Our results now indicate that imbalances in cellular metabolism and ACE2 expression in kidney organoids due to elevated glucose levels directly lead to higher viral loads upon infection, potentially leading to a switch from an OXPHOS to an aerobic glycolytic state that could further contribute to higher susceptibility to SARS-CoV-2 infection. Similar results were observed in kidney proximal tubular cells isolated from diabetic patients, which exhibited increased altered mitochondrial respiration and enhanced glycolysis that correlated with higher SARS-CoV-2 infections compared with non-diabetic samples. Interestingly, the exposure of kidney patient cells to a metabolic modulator that boosts mitochondrial OXPHOS at the expense of glycolysis resulted in decreased SARS-CoV-2 infection, suggesting that both ACE2 expression and altered metabolism affect the outcome of SARS-CoV-2 infection in human kidney cells, which is in line with findings in our ACE2 re-expression experiments.

Overall, our results provide insights into the identification of diabetic-induced metabolic programming as a critical event that increases the susceptibility of kidney cells to SARS-CoV-2 infection. We hope that our observation will open the door to the identification of new interventions in COVID-19 pathogenesis targeting energy metabolism.

Limitations of study

Future work will be needed to address limitations of our study. (1) Utilizing more complex culture systems, including the assembly of a vascular component to kidney organoids, would help to further explore the impact of a high oscillatory glucose regimen in a more physiologically relevant context while also allowing us to better mirror diabetic endothelial cell dysfunction in the renal context. (2) The design of our studies focused on the early

(C) SARS-CoV-2 mRNA expression levels of mock or SARS-CoV-2-infected WT and ACE2 KO kidney organoids in control or diabetic conditions by qPCR. Data are mean $\pm$ SD. $n = 1$ independent experiment from a pool of 12 organoids/group with two technical replicates each.

(D) ACE2 mRNA expression levels of mock or SARS-CoV-2-infected WT and ACE2 KO kidney organoids in control or diabetic conditions by qPCR. Data are mean $\pm$ SD. $n = 1$ independent experiment from a pool of 12 organoids/group with two technical replicates each.

(E) Protein levels of ACE2 in WT and ACE2 KO kidney organoids under control or diabetic conditions by western blot analysis. β -actin was used as loading control. Data from a pool of 12 organoids/group are shown.

(F) Lentiviral transduction of ACE2 in ACE2 KO kidney organoids (ACE2t) under control or diabetic conditions. Immunofluorescence of mock or SARS-CoV-2-infected specimens at 1 dpi for the viral nuclear protein (NP, red), ACE2 (green), LTL (white), and DAPI (blue). Scale bars, 250 and 50 μ m (magnified views). $n = 1$ organoid (mock) and $n = 2$ organoids (SARS-CoV-2 infected).

(G) Quantification of ACE2 expression (shown as integrated density-IntD) in (F). Data are mean $\pm$ SD. $n = 2$ organoid per condition performing two technical replicates. No statistically significant differences were observed. One-way ANOVA, Tukey's multiple comparisons test.

(H) Quantification of the area of NP⁺ cells in (F). Data are mean $\pm$ SD. $n = 2$ organoid per condition performing two technical replicates. * $p < 0.05$, one-way ANOVA, Tukey's multiple comparisons test.

See also Data S1.

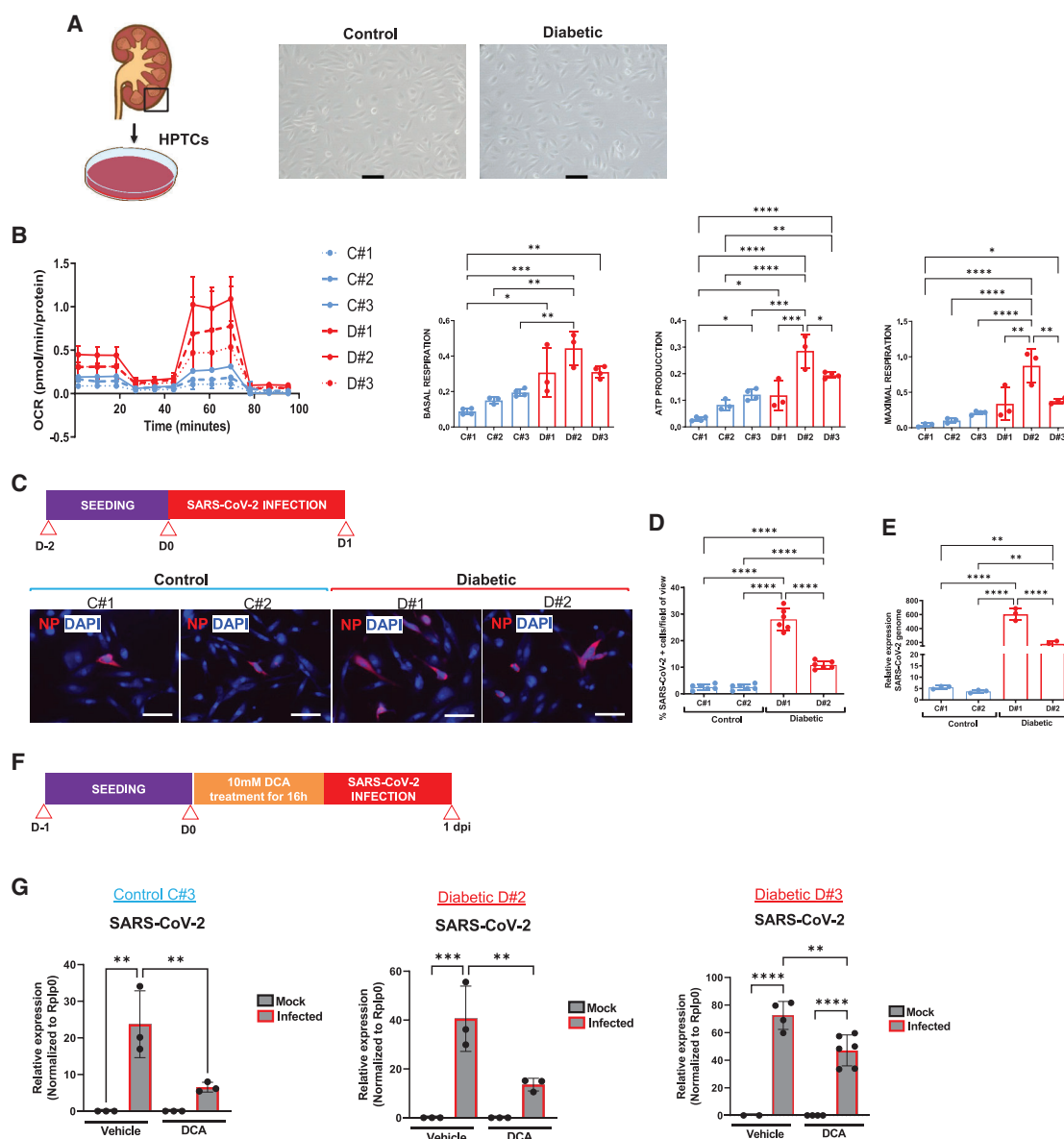


Figure 7. SARS-CoV-2 infection in tubular epithelial cells derived from diabetic human kidney biopsies

(A) Representative bright-field images of HPTCs from non-diabetic (control) or diabetic patient kidney biopsies. Scale bars, 100 μ m.

(B) Seahorse analysis of control and diabetic HPTCs. The oxygen consumption rate (OCR) data are normalized to total protein. Basal respiration, cellular ATP production, and maximal respiration are shown. Data are mean $\pm$ SD from at least $n = 3$ biological replicates/group. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$, one-way ANOVA, Tukey's multiple comparisons test.

(C) Experimental scheme for SARS-CoV-2 infection in control or diabetic HPTCs. Immunofluorescence of infected control or diabetic HPTCs at 1 dpi for the viral nuclear protein (NP, red) and DAPI (blue). Scale bars, 100 μ m.

(D) Quantification of NP⁺ cells in (C). Data are mean $\pm$ SD. $n = 2$ independent biological replicates per condition performing at least six technical replicates. **** $p < 0.0001$, one-way ANOVA, Tukey's multiple comparisons test.

(E) qPCR analysis of SARS-CoV-2-infected control or diabetic HPTCs at 1 dpi for the detection of SARS-CoV-2 mRNA. Data are mean $\pm$ SD. $n = 2$ independent biological replicates per condition with at least three technical replicates. ** $p < 0.01$; **** $p < 0.0001$, one-way ANOVA, Tukey's multiple comparisons test.

(F) Experimental scheme for SARS-CoV-2 infection in control or diabetic HPTCs treated with DCA or vehicle for 16 h prior infection.

(G) qPCR analysis of mock or SARS-CoV-2-infected control or diabetic HPTCs exposed to DCA or vehicle at 1 dpi. Data are mean $\pm$ SD. $n = 1$ independent experiment with at least two technical replicates. ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$, one-way ANOVA, Tukey's multiple comparisons test.

See also Figure S9.

stages of infection. As such, we cannot make any predictions with respect to the observed changes at later stages of the disease process. In this regard, inflammation represents a complex network of pathways that are influenced by external processes, which currently cannot be simulated in our model. (3) We did not study pancreatic organoids, and the pancreas is one of the major metabolic organs that could explain later complications of COVID-19.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cmet.2022.04.009>.

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AUTHOR CONTRIBUTIONS

This study was conceived and designed by N.M. N.M. and J.M.P. wrote the paper. E.G. and P.P. performed all cell culture experiments in wild-type cell lines. E.G. and P.P. performed all cell culture experiments in genome-edited cell lines. E.G. and P.P. performed all histological analysis. E.G. performed TEM analysis. E.G., P.P., A.M., G.J., A.L., A.H., R.O., D.M.-R., C.T., P.D.-P., O.H.A., J.M.P., and N.M. performed organoid data analysis including qPCR, western blot, and image quantification. M.L.S., V.M., S.B., and A.M. performed all the experiments involving SARS-CoV-2, including isolation, and helped with manuscript editing. F.G. and A.M. performed CRISPR-Cas9 lines. M.L.S., S.B., and A.V.-Z. prepared scRNA-seq samples. A.U.-A., J.P.R., and F.P. performed all the single-cell data analysis on genome-edited derived organoids. A.G. contributed to Seahorse analysis in LTL⁺ cells and HPTCs. C.H.P. performed one cell culture experiment to isolate LTL⁺ cells from kidney organoids for seahorse analysis and performed experiments to detect COL3A1, COL4A1, and PGC1 α by qPCR in kidney organoids and western blot to detect ACE2 and NPR1. C.H.P. performed immunofluorescence analysis to detect PODXL and LTL in kidney organoids in Figure S1A and PGC1 α , LTL, and NaK (sodium potassium ATPase) in LTL cells in Figure S2D. D.M.-R., P.-V.A., and J.M.C. performed patient cell isolation and characterization.

DECLARATION OF INTERESTS

A patent has been submitted to use human organoids to study SARS-CoV-2 infections and possibly develop new therapies. J.M.P. is a shareholder of Apeiron Biologics, which is developing ACE2 decoys for COVID-19 therapy.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human ACE-2	Bio-Techne R&D Systems S.L	Cat# AF933-SP; RRID: AB_355722
Recombinant Anti-Neuropilin 1 antibody	Abcam	Cat# ab81321; RRID: AB_1640739
α -Tubulin	Abcam	Cat# ab4074; RRID: AB_2288001
β -Actin	Sigma Aldrich	Cat# A5316; RRID: AB_476743
IRDye 800CW donkey anti-Goat IgG Secondary Antibody	Licor	Cat# 925-32214; RRID: AB_2687553
IRDye 800CW Goat anti-Rabbit IgG Secondary Antibody	Licor	Cat# 925-32211; RRID: AB_2651127
Human ACE2	Bio-Techne R&D Systems	Cat# AF933; RRID: AB_355722
Laminin	Merck	Cat# L9393; RRID: AB_477163
Collagen I	Abcam	Cat# ab34710; RRID: AB_731684
Collagen IV	Merck	Cat# AB769; RRID: AB_92262
Fibronectin	Abcam	Cat# ab2413; RRID: AB_2262874
Lotus Tetragonolobus Lectin (LTL), Biotinylated	Vector laboratories	Cat# B-1325; RRID: AB_2336558
Podocin	Merck	Cat# P0372; RRID: AB_261982
E-Cadherin	BD Bioscience	Cat# 610181; RRID: AB_397580
Human Podocalyxin Biotinylated	R&D Systems	Cat# BAF1658; RRID: AB_356080
SARS-CoV/SARS-CoV-2 Nucleocapsid	Abyntek Biopharma	Cat# 40143-MM05; RRID: AB_2827977
CD147	Abcam	Cat# ab666; RRID: AB_305632
Human Nephhrin	R&D Systems	Cat# AF4269 RRID: AB_2154851
Recombinant PE Anti-Sodium Potassium ATPase	Abcam	Cat# ab209299
PGC1 α	R&D Systems	Cat# NBP1-04676; RRID: AB_1522118
CD31	Abcam	Cat#ab28364; RRID: AB_726362
MEIS1/2/3	Thermo Fisher	Cat#39795; RRID: AB_2750570
Anti-Goat Alexa Fluor 488-conjugated	Jackson ImmunoResearch	Cat# 705-545-147; RRID: AB_2336933
Anti-Goat IgG Alexa Fluor 555-conjugated	Fisher Scientific	Cat# A-21432; RRID: AB_2535853
Anti-rabbit IgG Alexa fluor 488-conjugated	Fisher Scientific	Cat# A21206; RRID: AB_2535792
Anti-rabbit IgG Alexa fluor 555-conjugated	Fisher Scientific	Cat# A-31572; RRID: AB_162543
Anti-Mouse IgG CyTM3-conjugated	Jackson ImmunoResearch	Cat# 715-165-151; RRID: AB_2315777
Anti-Sheep IgG Alexa Fluor 555-conjugated	Fisher Scientific	Cat# A-21436; RRID: AB_2535857
Dylight 649 Streptavidin	Vector Labs	Cat# SA-5649; RRID: AB_2336421
Fluorescein-conjugated LTL	Vector Labs	Cat# FL-1321; RRID: AB_2336559
Anti- human ACE2-APC	LS Bio	Cat# LS-C275129
Normal Rabbit IgG APC-conjugated Control	R&D systems	Cat#. IC105A; RRID: AB_10174791
Recombinant Anti-Wilms Tumor Protein antibody [CAN-R9(IHC)-56-2]	N/A	Cat# ab89901; RRID: AB_2043201
Biological samples		
Human kidney tubular cells	N/A	N/A
Chemicals, peptides, and recombinant proteins		
Essential 8 medium	ThermoFisher	Cat# A1517001
Vitronectin	ThermoFisher	Cat# A14700
Trypan blue solution	Sigma	Cat# T8154
EDTA solution	ThermoFisher	Cat# 15575-038
Accumax	Stem Cell Technologies	Cat# 07921

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RPMI 1640	ThermoFisher	Cat#21875-034
Advanced RPMI 1640	ThermoFisher	Cat#12633020
GlutaMAX (200 mM)	ThermoFisher	Cat# 35050-038
Penicillin/Streptomycin	ThermoFisher	Cat# 15140122
Dulbecco's Modified Eagle Medium (DMEM) glucose-free	ThermoFisher	Cat# 11966025
D-Glucose	Merck	Cat# G7021
D-Manitol	Merck	Cat# M9647
ITS	Merck	Cat#I3146
Fetal Bovine Serum (FBS)	Gibco	Cat# 10270-106
Human EGF	R&D systems	Cat# 236-EG-01M
CHIR99021	Merck	Cat#SML1046; CAS: 2 52917-06-9
Recombinant human FGF9	PeproTech	Cat# 100-23
Heparin	Merck	Cat# H3149; CAS: 9041-08-1
Activin A	R&D systems	Cat# 338-AC-050
Cell culture grade distilled water	ThermoFisher	Cat# 15230-089
Protease inhibitor cocktail	Roche	Cat#11836153001
Phosphate buffered saline (PBS) pH 7.4 (1x)	ThermoFisher	Cat#1001-015
RIPA buffer	Cell signaling	Cat#9806
4-15% Mini-PROTEAN TGX Precast Protein Gels	BioRad	Cat# 4561083
SYBR Green PCR Master Mix	Applied Biosystem	Cat# KK4605
Fluoromount-G	Southern Biotech	Cat# 0100-01
Triton X-100	Sigma	Cat# T8787
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	ThermoFisher	Cat# D1306
DNA dye Draq5	Abcam	Cat# ab108410
Dimethyl Sulfoxide (DMSO)	Merck	Cat# D2650; CAS: 67-68-5
Paraformaldehyde	Anane	Cat# sc-281692
Donkey serum	Millipore	Cat# S30
Glutaraldehyde	Sigma-Aldrich	Cat# G7776
Sodium phosphate dibasic dihydrate $\geq 99.0\%$	Sigma-Aldrich	Cat# 71643
Sodium phosphate monobasic $\geq 98\%$	Sigma-Aldrich	Cat# S3139
Trizol	ThermoFisher	Cat# 15596018
Chloroform	Sigma Merck	Cat# 1.02445 CAS:67-66-3
2-Propanol	Panreac	Cat# 131090 CAS: 67-63-0
Ethanol	VWR	Cat# 100983.2500; CAS: 64-17-5
Actinomycin D	Sigma	Cat# A9415-5MG
Nuclease-Free Water	Ambion	Cat# AM9937
Lipofectamine RNAiMAX Transfection Reagent	ThermoFisher	Cat# 13778150
Dichloroacetic acid	Merck	Cat# 347795-10G
Renal Epithelial Cell Growth Medium	Lonza	Cat# CC-3190
Intercept (TBS) Blocking Buffer	LICOR	Cat# 927-60001
Critical commercial assays		
BCA Protein Assay Kit	ThermoFisher	Cat# 23225
cDNA Reverse Transcription Kit	Applied Biosystems	Cat# 4368813
Seahorse XFe96 FluxPak mini	Agilent Technologies	Cat# 102601-100
Chromium Single Cell 3' Library & Gel Bead Kit V3	10X Genomics (USA)	Cat# PN-1000075
NSQ 500/550 Hi Output KT v2.5 (75 CYS)	Illumina (San Diego, CA 92122 USA)	Cat# 20024906
Streptavidin/Biotin blocking kit	Vector laboratories	Cat# SP-2002

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
scRNA seq data kidney organoids	This study	GEO: GSE181002
Data S1 Source Data	This study	https://doi.org/10.17632/3m6gs9hfsd.1
CRISPResso2parser.py	open source	https://doi.org/10.5281/zenodo.6457752 ; https://github.com/amarcog/CRISPResso2parser.py
Experimental models: Cell lines		
ES[4] Human Embryonic Stem Cell line	The National Bank of Stem Cells (ISCIII,Madrid)	https://www.isciii.es/
CBiPS1sv-4F-40	The National Bank of Stem Cells (ISCIII,Madrid)	https://www.isciii.es/
Vero E6	ATCC CRL 1586	N/A
Oligonucleotides		
crRNA sequences	This paper	N/A
ACE2ko Cr1 (5'-3') TAGACTACAATGAGAGGCTC	N/A	N/A
ACE2ko Cr2 (5'-3') GCCATTATATGAAGAGTATG	N/A	N/A
BSGko Cr1 (5'-3') CTTGGAGCCAAGGTCTTCTA	N/A	N/A
BSGko Cr2 (5'-3') TTCCTACCGTAGAAGACCT	N/A	N/A
NRP1ko_Cr1 (5'-3') GGAATTTGAAAGCTTTGACC	N/A	N/A
NRP1ko_Cr2 (5'-3') GGGGACTTTATCACTCCACT	N/A	N/A
MiSeq oligos	This paper	N/A
MiSeq_ACE2ko_F (5'-3') ACACTCTTTCCCTACACGA CGCTCTTCCGATCTTGCTGTGCTTTGGGATAACAGGT	N/A	N/A
MiSeq_ACE2ko_R (5'-3') GACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCACACAGAGAGCTTCAGG	N/A	N/A
MiSeq_BSGko_F (5'-3') ACACTCTTTCCCTACACGA CGCTCTTCCGATCTGGGGAGGAGCCGACAGGTTTC	N/A	N/A
MiSeq_BSGko_R (5'-3') GACTGGAGTTCAGACGTG TGCTCTTCCGATCTCGTCTCCTTCAGCACCACG	N/A	N/A
MiSeq_NRP1ko_F (5'-3') TGGGGAAGTTGTT TAAGTGGGA	N/A	N/A
MiSeq_NRP1ko_R (5'-3') ATCCATCCCAGAT TTCTAGCCG	N/A	N/A
qPCR oligos	This paper	N/A
SARS-CoV-2 (oligo1)_Forward GCCTCTTCTC GTTCTCATCAC	Eurofins	N/A
SARS-CoV-2 (oligo1)_Reverse AGCAGCATCA CCGCCATTG	Eurofins	N/A
SARS-CoV-2 (oligo2)_Forward AGCCTCTTCT CGTTCCTCATCAC	Eurofins	N/A
SARS-CoV-2 (oligo2)_Reverse CCGCCATTG CCAGCCATTG	Eurofins	N/A
TMPRSS2_Foward GTCCCCACTGTCTACGAGGT	Thermo Fisher	N/A
TMPRSS2_Reverse CAGACGACGGGTTGGAAG	Thermo Fisher	N/A
NRP1_Foward GGCCTTTTTGCAACGATAAA	Thermo Fisher	N/A
NRP1_Reverse TCGCATTTTTCACTTGGGTGAT	Thermo Fisher	N/A
BSG_Foward CCGCAACCACCTTACTCG	Thermo Fisher	N/A
BSG_Reverse GGACAGAGGTTTGATGGTG	Thermo Fisher	N/A
ACE2_Foward CGAAGCCGAAGACCTGTTCTA	Thermo Fisher	N/A
ACE2_Reverse GGGCAAGTGTGACTGTTCC	Thermo Fisher	N/A
HK2_Foward AGCCCTTTCTCCATCTCCTT	Thermo Fisher	N/A
HK2_Reverse AACCATGACCAAGTGCAGAA	Thermo Fisher	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
LDHA_Foward GGAGATCCATCATCTCTCCC	Thermo Fisher	N/A
LDHA_Reverse GGCCTGTGCCATCAGTATCT	Thermo Fisher	N/A
PGC1 α _Forward CTGCTAGCAAGTTTGCCTCA	Thermo Fisher	N/A
PGC1 α _Reverse AGTGGTGCAAGTGACCAATCA	Thermo Fisher	N/A
COL3A1_Foward AGGACTGACCAAGATGGGAA	Thermo Fisher	N/A
COL3A1_Reverse AGGGGAGCTGGCTACTTCTC	Thermo Fisher	N/A
COL4A1_Foward CCTTTTGTCCCTTCACTCCA	Thermo Fisher	N/A
COL4A1_Reverse CTCCACGAGGAGCACAGC	Thermo Fisher	N/A
RPLP0_Foward CCATTCTATCATCAACGGGTACAA	Thermo Fisher	N/A
RPLP0_Reverse AGCAAGTGGGAAGGTGTAATCC	Thermo Fisher	N/A
SLC16A1_Foward GGCTGTCTGTATGGTGGAG	Thermo Fisher	N/A
SLC16A1_Reverse GACAAGCAGCCACCAACAATC	Thermo Fisher	N/A
PODXL_Foward GATAAGTGCGGCATACGGCT	Thermo Fisher	N/A
PODXL_Reverse GCTCGTACACATCCTTGGCA	Thermo Fisher	N/A
WT1_Foward GCCAGGATGTTTCCTAACGC	Thermo Fisher	N/A
WT1_Reverse CGAAGGTGACCGTGCTGTAA	Thermo Fisher	N/A
NPHS1_Foward GGCTCCCAGCAGAACTCTT	Thermo Fisher	N/A
NPHS1_Reverse CACAGACCAGCAACTGCCTA	Thermo Fisher	N/A
SLC3A1_Foward CACCAATGCAGTGGGACAAT	Thermo Fisher	N/A
SLC3A1_Reverse CTGGGCTGAGTCTTTTGGAC	Thermo Fisher	N/A
MAFB_Foward GACGCAGCTCATTGAGCAG	Thermo Fisher	N/A
MAFB_Reverse CTCGCACTTGACCTGTAGGC	Thermo Fisher	N/A
Endoglin_Foward CCTACGTGCTCCTGGCTCATC	Thermo Fisher	N/A
Endoglin_Reverse GGTGTGTCTGGGAGCTTGAA	Thermo Fisher	N/A
Meis1_Foward GGGCATGGATGGAGTAGGC	Thermo Fisher	N/A
Meis1_Reverse GGGTACTGATGCGAGTGCAG	Thermo Fisher	N/A
Vimentin_Foward GTTGACAATGCGTCTCTGG	Thermo Fisher	N/A
Vimentin_Reverse TGTTCTGAATCTGAGCCTG	Thermo Fisher	N/A
VEGFR_Foward CACATTGGCCACCATCTGAAC	Thermo Fisher	N/A
VEGFR_Reverse CCATCAGAGGCCCTCCTTG	Thermo Fisher	N/A
PDFGR α _Forward GAGCGCTGACAGTGGCTACAT	Thermo Fisher	N/A
PDFGR α _Reverse TCGTCCTCTCTCTGATGAAGGT	Thermo Fisher	N/A
PDK1_Foward GGATTGCCCATATCACGTCTTT	Thermo Fisher	N/A
PDK1_Reverse TCCCGTAACCCTCTAGGGAATA	Thermo Fisher	N/A
PDK2_Foward ATGAAAGAG ATCAACCTGCTTCC	Thermo Fisher	N/A
PDK2_Reverse GGCTCTGGACATACCAGCTC	Thermo Fisher	N/A

Plasmids

psPAX2	Addgene	Cat#12260; https://www.addgene.org/12260/
pMD2.G	Addgene	Cat#12259; https://www.addgene.org/12259/
pLENTI_hACE2_PURO	Addgene	Cat#155295; https://www.addgene.org/155295/

Software and algorithms

Fiji ImageJ2 version 2.3.0	NIH	https://imagej.net/software/fiji/
Prism 5	Graphpad Software	https://www.graphpad.com/scientific-software/prism
Image Studio Lite Version 5.2 software	LICOR	https://www.licor.com/bio/image-studio-lite/
FlowJo Software	FlowJo	N/A
Cell Ranger 4.0.0	10x Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/downloads/latest

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Seurat R package 3.2.1	open source	https://satijalab.org/seurat/
fgsea R package 1.16.0	open source	http://bioconductor.org/packages/release/bioc/html/fgsea.html
enrichR R package v3.0	open source	https://CRAN.R-project.org/package=enrichR

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nuria Montserrat (nmontserrat@ibecbarcelona.eu).

Materials availability

All unique organoids generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability

- Raw sequencing data for the single cell kidney organoid reported in this paper were deposited in Gene Expression Omnibus (GEO) under the accession number GEO: GSE181002.
- Original code has been deposited at Zenodo and is publicly available at <https://doi.org/10.5281/zenodo.6457752>.
- All values used to generate the graphs of the paper, original western blot images and additional microscopy images can be found in the file [Data S1](#) that is also available from Mendeley Data at <https://doi.org/10.17632/3m6gs9hfsd.1>. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

hPSC lines were obtained after the approval of the Ethics Committee from the Clinical Translational Program for Regenerative Medicine in Catalonia (P-CMR [C]) and the Comisión de Seguimiento y Control de la Donación de Células y Tejidos Humanos del Instituto de Salud Carlos III (project numbers: 0336E/1517/2015; 0336E/4166/2020; 0336E/4168/2020; 0336E/1124/2021/ 0336E/2723/2021). hESC ES[4] line and CBiPS1sv-4F-40 iPSC line were obtained from The National Bank of Stem Cells (ISCIII, Madrid). Both hPSC lines were maintained and grown in Essential 8 medium (A1517001, ThermoFisher) in cell culture plates coated with 5 µg/mL vitronectin (A14700, ThermoFisher) with 5% CO₂ at 37 °C. Cells were passaged every 4–6 days by disaggregating hPSC colonies into small cell clusters using 0.5 mM EDTA (15575-038, ThermoFisher). Vero E6 (ATCC CRL 1586) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS; 10270-106, Gibco) and 1% penicillin/streptomycin (15140122, ThermoFisher). Primary renal proximal tubular epithelial cells were isolated as previously described (Baer and Geiger, 2008; Baer et al., 1997) upon ethics committee of Hospital Clinic de Barcelona approved the procedure (project number: HCB/2021/0119).

Renal proximal tubular epithelial cells were isolated from healthy renal tissues after nephrectomy of renal cell carcinomas. Briefly, prior to cell isolation, the fibrous capsule and the inner medulla were removed and the pieces of tissue (1-mm²) were digested for 1 h with agitation at 37 °C in Iscove's Modified Dulbecco's Medium containing 1% collagenase IV (Invitrogen). The digested tissue fragments were filtered in 300-, 100-, and 70-µm cell strainers (BD Biosciences), and cell suspensions were overlaid on a pre-cooled Percoll density gradient solution (starting density 1.07 g/ml, Amersham Biosciences) and centrifuged for 40 min at 4 °C at 16,000 rpm. This procedure established a gradient with densities between 1.019 and 1.139 g/ml. The fraction between 1.05 and 1.076 g/ml was collected and washed three times in three volumes of cold Hanks' buffered saline solution (Invitrogen). Finally, renal proximal tubular epithelial cells were plated on plastic plates in proximal tubular cell medium consisting of DMEM without glucose (11966025, ThermoFisher) supplemented with 5% FBS (Gibco), 5 mM glucose (Merck), penicillin/streptomycin (15140122, ThermoFisher), ITS 1X (I3146, Merck), and 40 ng/mL human EGF (236-EG-01M, R&D systems) for expansion. Cells were also cultured in REGM medium (CC-3190, Lonza) when indicated.

SARS-CoV-2 isolate

SARS-CoV-2 were isolated on Vero-E6 cells from nasopharyngeal sample of patient diagnosed with COVID-19 in Sweden as described before in Monteil et al. (2020). Alternatively, SARS-CoV-2 (strain BavPat1) was obtained via the European Virology Archive. Virus were amplified in Vero E6 cells and titrated using a plaque assay as previously described (Becker et al., 2008) with fixation of cells 72 hours post infection. Virus was used at a passage 3.

METHOD DETAILS

Generation of genome edited hPSC lines

ACE2 and *BSG* knockout lines were generated using engineered ES[4] and CB[40] PSC lines, in which a doxycycline-inducible Cas9 cassette was introduced at the AAVS1 safe harbor locus (Marco, 2019, 2020). Two crRNAs per gene were designed using the Alt-R Custom Cas9 crRNA Design Tool (IDT) (key resources table). IDT crRNA/tracrRNA duplexes were annealed following manufacturer's guidelines and transfected using Lipofectamine RNAiMAX Transfection Reagent (13778150, ThermoFisher) at 10 nM final concentration in 0.25 million doxycycline-treated ES[4] or CB[40] PSCs/mL. Cells were grown 3–5 days after transfection and single-cell seeded at 10, 20, 50, 100 cells/cm². Leftover cells were used for DNA purification and further pool editing analysis. Clonal lines were established by manual colony picking (48 clones/crRNA) and further replica-plated. One replica was stored in nitrogen, while the other was used for DNA purification and MiSeq analysis of a locus-specific pooled PCR library generated through two rounds of PCR introducing Illumina adapters and specific indexes labeling each clone (key resources table). Indel frequency in edited pools and allelic composition of clones was obtained by analyzing trimmed Fastq reads using CRIPRESSO2 webtool (Clement et al., 2019). Analyzed clones were automatically genotyped based on CRIPRESSO2 results by our in-house CRIPRESSO2parser.py scripts (available at <https://doi.org/10.5281/zenodo.6457752>). For each crRNA edit, at least one non-edited clone (+/+), one heterozygous clone (+/frameshift, or +/-fs), and one homozygous or trans-heterozygous clone (fs/fs) were amplified, stocked, and used for further experiments.

Kidney organoid differentiation

Prior differentiation (day -1), undifferentiated hPSCs colonies were dissociated into cell clumps using 0.5 mM EDTA (15575-038ThermoFisher) at 37°C for 3 minutes. To obtain single cell suspensions samples were then incubated in Accumax (07921, Stem Cell Technologies) at 37°C for 3 additional minutes. Cells were counted using the Countess Automated Cell Counter (Invitrogen) and seeded at 100,000 cells/well on 24 multi-well plates coated with 5 µg/mL vitronectin in the presence of Essential 8 medium (A1517001, Life Technologies) at 37°C overnight. The next day (day 0), the differentiation was initiated by treating monolayer cultures with 8 µM CHIR99021 (CHIR; SML1046, Merck) in Advanced RPMI medium consisting of Advanced RPMI 1640 basal medium (12633020, ThermoFisher) supplemented with 1% Penicillin-Streptomycin and 1% of GlutaMAX (35050061, ThermoFisher) for 3 days with daily medium changes. On day 3, monolayer cultures were treated with 200 ng/mL FGF9 (100-23, Peprotech), 1 µg/mL heparin (H3149, Merck) and 10 ng/mL activin A (Act A) (338-AC-050, Vitro) in Advanced RPMI 1640 basal medium supplemented with 1% Penicillin-Streptomycin and 1% of GlutaMAX for 1 day. On day 4, cell monolayers were treated with 5 µM CHIR, 200ng/mL FGF9 and 1 µg/mL Heparin Advanced RPMI 1640 basal medium (12633020, ThermoFisher) supplemented with 1% Penicillin-Streptomycin and 1% of GlutaMAX for 1 hour. After this 1 hour-treatment cell monolayers were dissociated using TrypLE Express Enzyme (1260402, ThermoFisher) for 1 min collected, and counted. Next, 100,000 cells/well were dispensed on a V-shape 96 multi-well plate (249935, ThermoFisher) and centrifuged at 300g for 5min. Cell spheroids were cultured in Advanced RPMI 1640 basal medium supplemented with 1% Penicillin-Streptomycin and 1% of GlutaMAX, 200ng/mL FGF9 and 1 µg/mL heparin for 7 days with medium changes every other day. From day 11, organoids were maintained in Advanced RPMI 1640 basal medium supplemented with 1% Penicillin-Streptomycin and 1% of GlutaMAX. From that stage medium was changed every other day.

Glucose challenge in kidney organoids

Normoglycemic conditions were emulated exposing kidney organoids to DMEM glucose-free (11966025, ThermoFisher) supplemented with 5 mM D-Glucose (G7021, Merck) changing medium every day until day 23. High oscillatory glucose conditions involved the exposure of day 16 kidney organoids to DMEM glucose-free (11966025, ThermoFisher) supplemented with 5mM D-Glucose (5 mM glucose medium) on even days or with 25mM of D-Glucose (25 mM glucose medium) on odd days until day 23 (5-25mM oscillatory glucose regime). 5 mM glucose medium was additionally supplemented with 20 mM D-Mannitol (M9647, Merck) to obtain the same molarity as in the 25 mM glucose medium.

Isolation of proximal tubular-like cells from kidney organoids

Kidney organoids were stained with fluorescein-conjugated LTL (FL-1321, Vector Laboratories) as described elsewhere (Garreta et al., 2019). Kidney organoids were then dissociated to single cells using Accumax (07921, Stem Cell Technologies) for 15 min followed by 0.25% (wt/vol) trypsin (25300-054, Life Technologies) for 15 min at 37 °C. SA3800 software version 2.0.4 (SONY) was used to acquire flow cytometry samples in the Sony SA3800 spectral cell analyzer (SONY). FACSDiva software version 8.0.1 (BD Biosciences) was used in the FACS Aria Fusion instrument (BD Biosciences) for cell sorting experiments. FlowJo software version 10 was used to analyze the data.

ACE2 mRNA half-life in kidney organoids exposed to 5mM and 5-25 mM glucose conditions

Kidney organoids were treated with 50 µg/ml actinomycin D (Sigma) to inhibit transcription. At the indicated times (0, 4, and 8 hours) total RNA was isolated from the kidney organoids (two pools of 12 kidney organoids per time point) and relative ACE2 mRNA levels were determined by RT-qPCR.

ACE2 lentiviral production and transduction of kidney organoids

Lentiviruses containing full length human ACE2 were produced by polyethylenimine (PEI) transfection of HEK 293T cells (4μg pMDG2, 4μg sPAX, and 8μg lentivirus of interest per well). Corresponding plasmids were purchased from Addgene (see [key resources table](#)). Three days post-transfection the lentivirus containing supernatants were collected, filtered with 0.45μm filters and pelleted at 20,000 rpm for 3 hours. The virus pellet was resuspended in 200μL of OptiMem, aliquoted and stored at -80 °C. 10⁶ lentivirus particles were added to each kidney organoid 3 day prior to infection. Lentiviruses were removed at the time of infection and media containing SARS-CoV-2 or fresh media was added to organoids for 1 hour. Following virus infection, media was removed, organoids were washed 2x and fresh media was added. 24h post-infection organoids were harvested for RNA, protein or fixed in 4% PFA for immunofluorescence.

SARS-CoV-2 infections of kidney organoids and proximal tubular kidney cells

Kidney organoids were infected with 10⁶ SARS-CoV-2 infectious particles (as determined in Vero cells) in Advanced RPMI medium (ThermoFisher) in a volume of 50μl per well of a 96-well ultra-low attachment plate for 1 hour. Proximal tubular cells were seeded in 24-well plates in REGM medium. One day post-seeding, cells were infected with 10⁶ MOI 1 (as determined in Vero cells) SARS-CoV-2 infectious particles in a final volume of 200μl per well in proximal tubular cell medium at 37°C for 1 hour followed by washing with PBS and adding 500μl of new proximal tubular cell medium. At the different days post-infection, samples (organoids or cells) were washed 3 times with PBS and then lysed using Trizol (ThermoFisher), RIPA buffer (ThermoFisher) or 4% paraformaldehyde (153799, Anamed) for ulterior analysis.

SARS-CoV-2 infections of proximal tubular kidney cells exposed to DCA

Proximal tubular cells were seeded in 24-well plates in proximal tubular cell REGM medium. One day post-seeding cells were exposed to 10 mM DCA for 16 hours. After treatments cells were washed with PBS and subsequently infected with SARS-CoV-2 infectious particles (MOI 1) in a final volume of 200μl of REGM medium at 37°C for 1 hour. Cells were washed with PBS and 500μl of new REGM medium was added. At 1 day post-infection, cells were washed 3 times with PBS and then lysed using Trizol (ThermoFisher) for further analysis.

Single-cell RNA sequencing

Single cell RNA sequencing was performed as described in our previous study ([Monteil et al., 2020](#)). Briefly, kidney organoids were collected and washed twice with PBS, and further dissociated into a single cell suspension by treating them with Accutase (07921, Stem Cell Technologies) for 15 min at 37°C followed by Trypsin-EDTA 0.25% (wt/vol) trypsin (25300-054, Life Technologies) for additional 15 min at 37°C. The reaction was deactivated by adding 10% FBS. The cell suspension was then passed through a 40μm cell strainer. After centrifugation at 1,000 RPM for 5 minutes cell numbers and viability were analyzed using Countess Automated Cell Counter (Invitrogen). Then cell suspensions were loaded onto a well of a 10x Chromium Single Cell instrument (10x Genomics). Barcoding and cDNA synthesis were performed according to the manufacturer's instructions. Qualitative analysis was performed using the Agilent Bioanalyzer High Sensitivity assay. The cDNA libraries were constructed using the 10x Chromium Single cell 3' Library Kit v3.1 according to the manufacturer's original protocol. Libraries were sequenced on either Illumina NovaSeq 6000 or NextSeq 500 2x150 paired-end kits using the following read length: 28bp Read1 for cell barcode and UMI, 8bp I7 index for sample index and either 94bp Read2 in the 5 mM, 11 mM and 5-25 mM oscillatory glucose samples (diabetic samples). For ACE2 WT and ACE 2KO samples (ACE2 samples) 55bp read length was used for Read2. Libraries were pre-processed using Cell Ranger (4.0.0) from 10X Genomics (<http://10xgenomics.com>). Reads from the ACE2 samples were aligned to the reference human genome (GRCh38), whilst diabetic samples were aligned to a custom reference built for this work including the SARS-CoV-2 genome (isolate Wuhan-Hu-1, NC_045512.2). Genome annotation corresponded to Ensembl v93, adding a single gene with a single exon spanning the entire SARS-CoV-2 sequence. The median number of unique molecular identifiers (UMIs) per cell was between 2,300 and 4,600, with a median of 1,300-2,200 genes detected per condition.

Protein extraction and western blot analysis in kidney organoids

Protein was extracted from kidney organoids cultured in 5 mM glucose, 5-25 mM oscillatory glucose or Advance RPMI with or w/o infection using RIPA buffer (ThermoFisher) supplemented with complete protease inhibitor cocktail (ThermoFisher). Samples were centrifuged at 13,000 g for 15 mins at 4°C. Supernatant were collected and protein concentration was measured using a bicinchoninic acid (BCA) protein quantification kit (Thermo Scientific). For western blot analyses, 25 μg of protein were separated in 4-15% Mini-PROTEAN TGX precast gel (BioRad) and blotted onto nitrocellulose membranes. Membranes were blocked at room temperature for 1 hour with TBS 1X-5% BSA. Membranes were then incubated in primary antibody (see [key resources table](#)) overnight at 4°C. The membranes were then washed with PBST (PBS 1X + 0.05% Tween20; Merck) for three times 5 minutes and incubated with the secondary antibody (see [key resources table](#)). After washing with PBST for 5 minutes twice and with PBS for 5 minutes once, membrane-bound antibodies were detected by fluorescence with the Odyssey Fc Imaging System. Beta-actin (1:5000, Sigma) or Alpha-tubulin (1:5000; Abcam) was used as a loading control for normalization and quantification. Images were analyzed with Image Studio Lite Version 5.2 software.

Histology and Immunocytochemistry

Specimens were fixed at the indicated time points with 4% paraformaldehyde (153799, Anamed) overnight at 4°C. Samples were then washed twice with PBS, embedded in paraffin and sectioned into 5 to 10 μ m samples. Sections were stained for Hematoxylin and Eosin, Masson's trichrome and Periodic acid–Schiff. Images were captured using the AF7000 Leica microscope.

Immunofluorescence samples were fixed with 4% paraformaldehyde (153799, Anamed) for 20 minutes at room temperature, washed three times with PBS and blocked using Tris-buffered saline (TBS) containing 6% donkey serum (S30, Millipore) and 1% Triton X-100 (T8787, Sigma) for 1 h at room temperature. Samples were treated overnight at 4°C with the primary antibodies indicated in [key resources table](#) diluted in antibody dilution buffer (TBS solution with 6% donkey serum and 0.5% Triton X-100). Samples were then washed three times with antibody dilution buffer and further incubated during 4 h at room temperature with fluorescent conjugated secondary antibodies indicated in [key resources table](#). For detection of LTL positive cells (LTL+), samples were stained with biotinylated LTL (B-1325, Vector Labs) using a streptavidin/biotin blocking kit (SP-2002, Vector Labs). LTL+ cells were detected using Alexa Fluor 488 conjugated with streptavidin (SA5488, Vector Labs). Nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI; 1:5000, D1306, Life Technologies) for 30 min. Samples were mounted in Fluoromount-G (0100-01, Southern Biotech) and visualized using a Zeiss LSM780 or LSM 880-Airyscan Elyra confocal microscope.

Electron microscopy

Specimens were fixed with 2.5% glutaraldehyde containing 1% tannic acid in 0.1 M phosphate buffer (PB, pH 7.4). Then samples were post-fixed for 1 hour at 4°C with 1% OsO₄ in 0.1 M PB. Epoxy resin embedding was performed after graded ethanol series. Then toluidine blue staining was performed in semithin sections using a light microscope. Finally, ultrathin sections were prepared using an EM UC7 ultramicrotome (Leica Microsystems, Mannheim, Germany) and collected on copper grids. Then 4% uranyl acetate and lead citrate were used for staining. Samples were subsequently analyzed with a JEM 1230 electron microscope (JEOL, UK, Ltd).

qRT-PCR

RNA was isolated from cells and kidney organoids using Trizol (Invitrogen). 2 μ g RNA was reverse transcribed using the cDNA archival kit (Life Technology), and qRT-PCR was run in the ViiA 7 System (Life Technology) machine using SYBRGreen Master Mix (Applied Biosystem) and gene-specific primers. The data were normalized and analyzed using the $\Delta\Delta$ Ct method. The primers sequences used are shown in [key resources table](#).

Oxygen consumption rate (OCR)

The measurement of OCR in kidney organoid-derived cells and proximal tubular cells was performed using an XF24 extracellular flux analyzer (Seahorse Bioscience) as previously described ([Dhillon et al., 2021](#); [Garreta et al., 2019](#)). Cells were plated at density of 20,000 cells/well in a Seahorse cell culture microplate in warm Seahorse XF Assay Medium supplemented with glucose 5mM, pyruvate 0.5mM and glutamine 2mM (Seahorse Bioscience). After 1 h of incubation at 37 °C, plates were loaded into an XF24 respirometry machine (Seahorse Bioscience). Uncoupled and maximum OCR were assayed with oligomycin (10 μ M) and FCCP (2 μ M). To inhibit complex I- and III-dependent respiration, rotenone (5 μ M) and antimycin A (15 μ M) were used, respectively. OCR represents the oxygen tension and acidification of the medium as a function of time (pmol min^{−1}). OCR was normalized to protein quantity in each well.

QUANTIFICATION AND STATISTICAL ANALYSIS

scRNA-Seq data analysis

The computational analysis of the resulting UMI count matrices was performed using the R package Seurat (3.2.1) ([Stuart et al., 2019](#)). Poor quality cells were removed, such as those in the lower and upper 2.5% quantiles for the number of UMIs and detected genes per cell in diabetic samples and those with < 1,000 detected genes per cell in ACE2 samples. For the latter, the upper thresholds were obtained from visual inspection of the UMI and detected genes distribution. In all cases cells with more than 10% of UMIs assigned to mitochondrial genes were discarded and genes expressed in less than 3 cells were removed from the analyses. Each dataset was subjected to normalization, identification of highly variable features and scaling using the *SCTransform* function, regressing out for the number of detected genes, the % of mitochondrial UMIs and cell cycle. All samples were integrated independently to remove batch effects among them and enable downstream comparisons between different conditions. The infection samples at differentiating conditions (11mM) were processed and integrated in the same way as 5mM and high oscillatory glucose conditions.

Principal component analysis was performed, and the top 20 components were kept for further analysis in the integrated ACE2 dataset. In the diabetic data set the top 40 component were kept for analysis. Clustering was performed by setting the resolution parameter to 0.6 in both cases Dimensional reduction for data visualization was done using the RunUMAP function.

Cell markers in each cluster were identified using the *FindConservedMarkers* and *FindAllMarkers* functions in the non-integrated counts by using the Wilcoxon Rank Sum test. Genes with p value < 0.05 (adjusted by Bonferroni's correction) were retained. Clusters were labelled by comparing the expression of the identified markers with publicly available databases located in KIT (Kidney Inter-active Transcriptomics) webpage [<http://humphreyslab.com/SingleCell/>] and with markers from previous publications ([Lindström et al., 2018a, 2018b](#)). Cell type annotations from the diabetic samples were transferred to the 11 mM analysis using the

TransferData function in Seurat. Differential expression analysis to identify changes across conditions were also performed using the Wilcoxon test, keeping as up-regulated genes those present in a fraction of 0.1 of either population, with a log fold-change greater than 0.1 and with an adjusted p value < 0.05. To perform over-representation analysis the R package enrichR ([Kuleshov et al., 2016](#)) was used with the database MSigDB_Hallmark_2020". Gene set enrichment analysis (GSEA) was done with the fgsea R package ([Korotkevich et al., 2021](#)), considering the hallmark gene sets from MSigDB.

Image analysis

Quantification of collagen fibers in Masson's trichrome staining of kidney organoids was performed as described elsewhere ([Chen et al., 2017](#)) using Fiji ImageJ2 version 2.3.0 software. Briefly, the color deconvolution plugin was used to generate monochromatic images from Masson's trichrome staining images. The integrated intensity of the green component corresponding to the collagen fibers was then calculated using ImageJ.

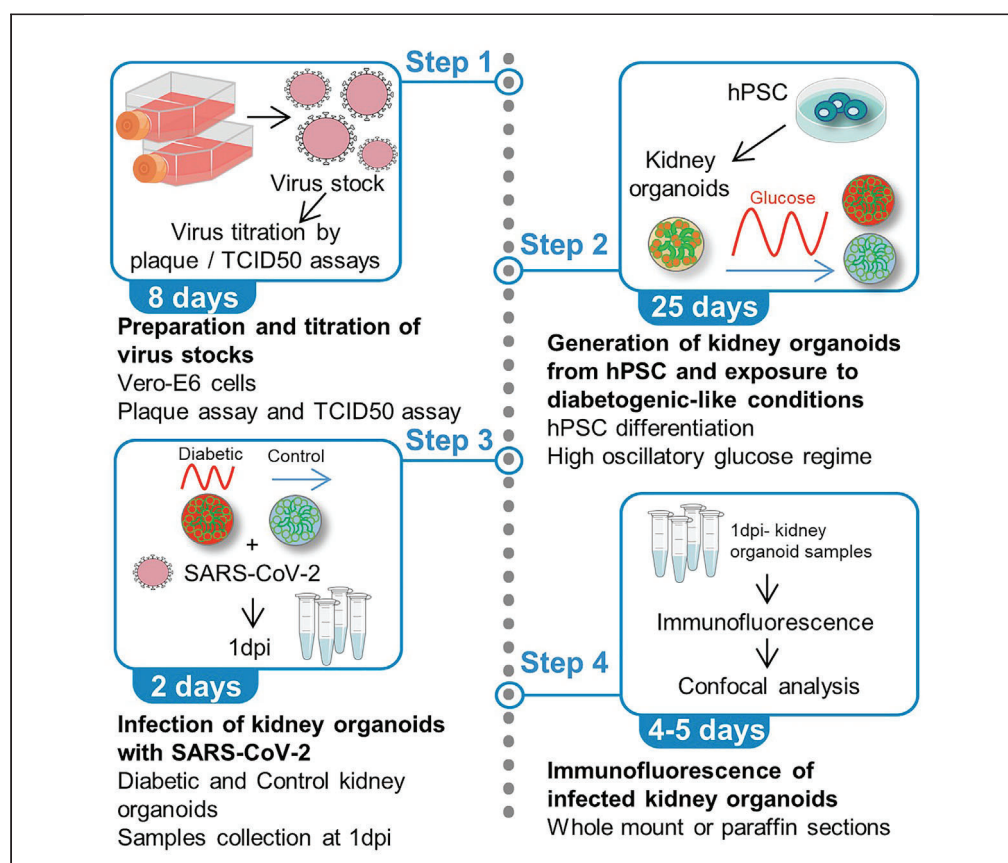
All raw confocal microscope images were processed with the Fiji ImageJ2 version 2.3.0 software. For optimal image visualization, Z-stacks were projected onto a tiff image where the background signal was removed, and the brightness contrast was enhanced. For fluorescent signal quantification, Z-stacks were projected as a sum of all image pixel grey values across the stack and then selection masks were created at each channel by the SetThreshold tool to measure area, intensity and integrated density of background-free signal. Colocalization was analyzed in Z-stacks using JACoP plugin under Fiji ImageJ2 version 2.3.0 software and calculated as changes of Manders' overlap coefficient.

Data representation and statistical analysis

Student's t test was used to analyze differences between two groups, and One-way or Two-way ANOVA was used to analyze inter-group differences (Tukey's multiple comparisons test). p values less than 0.05 were considered statistically significant. The analysis was performed using GraphPad Prism 5 (GraphPad software). Densitometry results of Western Blots were quantified using the Image Studio Lite Version 5.2 software. All data are presented as mean $\pm$ SD and other details such as the number of replicates and the level of significance is mentioned in figure legends and supplementary tables.

Protocol

Protocol for SARS-CoV-2 infection of kidney organoids derived from human pluripotent stem cells



This protocol presents the use of SARS-CoV-2 isolates to infect human kidney organoids, enabling exploration of the impact of SARS-CoV-2 infection in a human multicellular *in vitro* system. We detail steps to generate kidney organoids from human pluripotent stem cells (hPSCs) and emulate a diabetic milieu via organoids exposure to diabetogenic-like cell culture conditions. We further describe preparation and titration steps of SARS-CoV-2 virus stocks, their subsequent use to infect the kidney organoids, and assessment of the infection via immunofluorescence.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Description of technical details to generate kidney organoids from hPSCs

Approach to induce diabetic-like hallmarks in kidney organoids

Methodology to prepare and titrate SARS-CoV-2 virus stocks and infect kidney organoids

Evaluate viral infection in whole organoids or paraffin sections by immunofluorescence

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Protocol

Protocol for SARS-CoV-2 infection of kidney organoids derived from human pluripotent stem cells

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SUMMARY

This protocol presents the use of SARS-CoV-2 isolates to infect human kidney organoids, enabling exploration of the impact of SARS-CoV-2 infection in a human multicellular *in vitro* system. We detail steps to generate kidney organoids from human pluripotent stem cells (hPSCs) and emulate a diabetic milieu via organoids exposure to diabetogenic-like cell culture conditions. We further describe preparation and titration steps of SARS-CoV-2 virus stocks, their subsequent use to infect the kidney organoids, and assessment of the infection via immunofluorescence.

For complete details on the use and execution of this protocol, please refer to Garreta et al. (2022).¹

BEFORE YOU BEGIN

The protocol herein outlines the detailed procedures for using SARS-CoV-2 virus isolates to infect human kidney organoids. Specifically, we describe the steps to prepare SARS-CoV-2 virus stocks and determine virus titers using Vero-E6 Cells. We also detail the methodology to culture and differentiate hPSC into kidney organoids, and how to induce diabetic-like kidney organoids *in vitro*. Next, we explain how to efficiently infect human kidney organoids with SARS-CoV-2 and assess infection by immunofluorescence and confocal imaging.

△ **CRITICAL:** All laboratory procedures related to virus preparation, infection of cell cultures/organoids or collection of infected cells/organoids must be performed in certified



class II biological safety cabinets in a Biosafety Level 3 (BSL-3) containment laboratory. Personnel are required to wear appropriate personal protective equipment to protect from biological risks as determined by local requirements. Personal equipment as well as virus handling and decontamination of all materials must be performed before exiting the containment area. This includes decontamination of all tubes/samples that exit the BSL-3 area as well as ensuring that all protocols to collect RNA and protein are shown to kill any infectious virus. The protocols that state whether a virus has been inactivated must be tested, reviewed and approved by the competent Institutional Biosafety Committees or National agencies depending on the requirements at your institution.

Institutional permissions

SARS-CoV-2 can be isolated from patient samples with proper institutional approval. Additionally, SARS-CoV-2 variants can be obtained through international banks after confirmation that you are certified to have the proper BSL-3 containment to receive and propagate the virus. All approvals are Institute specific, and you should verify the local regulations prior to starting any infectious disease work. All experiments handling SARS-CoV-2 virus i.e., (but not limited to) isolation, culture, infection and inactivation must be separately reviewed and validated by the competent Institutional Biosafety Committees before the beginning of the project.

Culture of Vero-E6 cells

⌚ Timing: 1 week

This procedure describes the culture and expansion of Vero-E6 Cells and can be performed in BSL-1 or BSL-2 containment.

1. Supplement Dulbecco's Modified Eagle's Medium (DMEM) with 1% Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA), 10 mM HEPES Buffer Solution and 10% Fetal Bovine Serum (FBS) (Complete Growth Medium).
2. Pre-warm Complete Growth Medium at 37°C.
3. Thaw a cryovial with Vero-E6 Cells in a 37°C water bath.
 - a. Check the cryovial every 20 s. As soon as the cryovial content becomes liquid, transfer the cell suspension using a p1000 micropipette to a sterile 15 mL conical tube with 10 mL of pre-warmed Complete Growth Medium.
 - b. Centrifuge the cell suspension at 300 × g for 5 min.
 - c. Remove the supernatant and re-suspend the cell pellet in 10 mL pre-warmed Complete Growth Medium and carefully pipette up and down to create a homogeneous solution.
 - d. Count cells.
 - i. Mix thoroughly 10 µL of cell suspension and 10 µL of 0.4% Trypan Blue Stain, and pipette 10 µL of the mixture into a Countess™ cell counting chamber slide.

Note: Trypan Blue is used to discriminate live from dead cells. Dead cells will be stained blue and should not be counted.

- ii. Count viable cells using a Countess Automated Cell Counter.

Alternatives: A Neubauer cell counting chamber can be used to count viable cells under the microscope.

4. Dilute the cell suspension in Complete Growth Medium to have a cell density between 1.5–3.5 × 10⁵ viable cells/mL. Then, plate 15 mL of the cell suspension in a 75-cm² flask (3 × 10⁴ to 7 × 10⁴ cells/cm²).

Note: Vero-E6 Cells recovery improves by plating cells at high densities post-thaw (3×10^4 to 7×10^4 cells/cm²). Small 25-cm² or 50-cm² cell culture flasks can also be used for this purpose. For a 25-cm² flask, re-suspend the cells in 5 mL of Complete Growth Medium; for a 50-cm² flask, re-suspend the cells in 10 mL of Complete Growth Medium.

5. Incubate flasks at 37°C and 5% CO₂.
6. Split the cells when 80%–90% confluency is achieved using a split ratio of 1:10 (the dilution ratio depends on the cells needed for subsequent experiments. Use a split ratio 1:4 if you need cells 2–3 days after splitting. Use 1:10 if you need cells 7 days later or for on-going culture).
 - a. Remove medium and rinse cells with Phosphate Buffered Saline (PBS) 1×.
 - b. Remove the PBS 1× and add 1 mL (25-cm² flask) or 3 mL (75-cm² flask) of 0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA) solution.
 - c. Allow the flask to sit at 37°C to ensure cell detachment. This usually takes 3–5 min.
 - d. Quench the Trypsin-EDTA by adding an equal volume of fresh Complete Growth Medium.
 - e. Collect and pipette up and down to create a homogeneous solution and plate into new culture flasks.
 - f. Top up flask with extra Complete Growth Medium to reach a final volume of 10 mL for a 25-cm² or 15 mL for a 75-cm² flask.

Note: After recovery from frozen stock, Vero-E6 Cells need 2–3 passages to reach their regular growth rate.

Note: Prepare frozen stocks of Vero-E6 Cells from early passages. A confluent 75-cm² flask can be used to make 5 cryovials. To freeze cells, following trypsinization of a 75-cm² flask (see steps 6a–e above), add media back to cells to reach a total volume of 10 mL. Pellet cells by centrifugation at $300 \times g$ for 5 min. Remove the supernatant and add 5 mL of freezing medium consisting of FBS supplemented with 10% Dimethyl Sulfoxide (DMSO) to re-suspend the cell pellet. Transfer 1 mL of the cell suspension using a p1000 micropipette into each labelled cryovial. Immediately place the cryovials with the cells into a Mr Frosty™ Freezing Container and store at –80°C for 24 h before to stock them in liquid nitrogen.

Culture of human pluripotent stem cells

⌚ **Timing:** 2 weeks

This procedure describes the culture and expansion of hPSC using Complete Essential 8 (E8) Medium and Vitronectin (VTN-N) coated culture plates. Culture and expansion of hPSC should be performed in BSL-2 containment.

7. Prepare Complete E8 Medium.
 - a. Thaw E8 Supplement (50×) at 4°C for 12–16 h. Do not thaw at 37°C.
 - b. Add 10 mL of E8 Supplement and 5 mL of 10,000 U/mL Penicillin/Streptomycin (P/S) into 485 mL of E8 Basal Medium to obtain 500 mL of Complete E8 Medium.

Note: Complete E8 Medium can be stored at 2°C–8°C for up to 2 weeks. Before use, warm only the required amount of medium at 20°C–22°C. Do not warm the medium at 37°C.

8. Prepare VTN-N coated 6-well plates.
 - a. Thaw the VTN-N vial at 4°C. To avoid multiple freezing and thawing cycles prepare 60 µL VTN-N aliquots in sterile 1.5 mL Eppendorf tubes and freeze them at –80°C.

Note: 60 µL of VTN-N is the amount required to coat one 6-well plate.

- b. Dilute VTN-N 1:100 in Dulbecco's PBS (DPBS) 1× to have a VTN-N solution at 0.5 µg/mL.

Note: 60 µL of VTN-N is diluted in 6 mL of DPBS 1×.

- c. Place 1 mL of the VTN-N solution into each well of 6-well plate.
- d. Incubate at 20°C–22°C for 1 h.
- e. Before plating cells, aspirate the VTN-N solution from each well.

Note: VTN-N coated plates can be stored at 4°C for up to a week. Store the plates with the VTN-N solution. Do not allow drying. Prior to use, pre-warm the culture plate at 20°C–22°C for 1 h. Before plating cells, aspirate the VTN-N solution from each well.

Note: Other plate formats can be used to maintain and expand hPSC although 6 well-plates are recommended.

9. Prepare 0.5 mM EDTA in DPBS 1×.
 - a. Dilute 50 µL of 0.5 M EDTA in 50 mL of DPBS 1×.
 - b. Filter the obtained solution using a 0.22 µm pore size syringe filter.

Note: The 0.5 mM EDTA solution can be stored at 20°C–22°C for up to 6 months.

10. Thaw a cryovial containing hPSC in a 37°C water bath. [Troubleshooting 1](#).
 - a. After carefully submerging the cryovial in the water bath check the cryovial content every 20 s. As soon as the cryovial content becomes liquid, transfer the cell suspension using a p1000 micropipette to a sterile 15 mL conical tube containing 10 mL of Complete E8 Medium.
 - b. Centrifuge the cell suspension at 300 × g for 5 min. Remove the supernatant and gently re-suspend the cell pellet in 2 mL of Complete E8 Medium to obtain a homogeneous cell suspension.
 - c. Count cells.
 - i. Take 50 µL of cell suspension in a 1.5 mL Eppendorf tube.
 - ii. Add 150 µL of Accumax and incubate at 37°C for 5 min to obtain a single cell suspension.
 - iii. Mix 10 µL of the single cell suspension with 10 µL of 0.4% Trypan Blue Stain and pipette 10 µL of the mixture into a Countess™ cell counting chamber slide.
 - iv. Count viable cells using a Countess Automated Cell Counter.
 - v. Consider that the initial cell suspension is diluted 1:4 with Accumax. Thus, the final number of cells per mL is [Number of viable cells] × 4.

Alternatives: A Neubauer cell counting chamber can be used to count viable cells under the microscope.

11. Dilute the cells into the volume of Complete E8 Medium required for achieving a cell density between $4.75\text{--}5.7 \times 10^5$ viable cells/mL. Then, plate 2 mL of the cell suspension per well of VTN-N coated 6-well plate ($1\text{--}1.2 \times 10^5$ cells/cm²).

△ CRITICAL: When thawing hPSC, do not add ROCK inhibitor to the medium. hPSC routinely exposed to ROCK inhibitor may lead to inefficient generation of kidney organoids.

△ CRITICAL: After thawing, hPSC should be passaged at least twice before using the cells for subsequent differentiation methodologies.

12. Incubate the 6-well plate containing hPSC at 37°C and 5% CO₂. Refresh with Complete E8 Medium the day after cell plating. Change medium every other day.

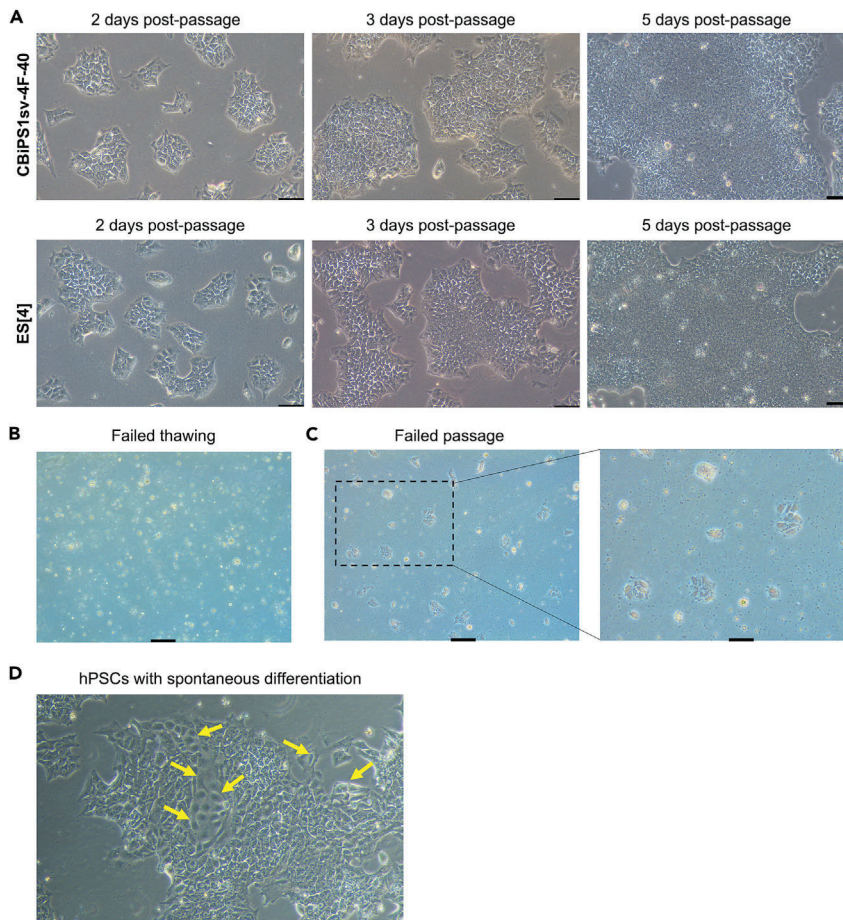


Figure 1. Typical morphology of hPSC colonies cultured on VTN-N-coated plates with E8 medium

(A) Representative bright field images of the hiPSC line CBiPS1sv-4F-40 and the hESC line ES[4] at day 2, day 3 and day 5 after passing. After 5 days in culture, hPSC colonies show nearly 80% confluency.
(B) Representative bright field image taken 24 h after thawing an hPSC cryovial that shows dead floating hPSCs because of a failed thawing procedure.
(C) Representative bright field image taken 48 h after hPSC passing that shows very few small cell clusters indicating a failed passage procedure.
(D) Representative bright field images of hPSC colonies with spontaneous differentiation. Yellow arrows indicate differentiated cells. Scale bars in (A–D), 100 μ m.

13. Routinely, passage hPSC when the colonies cover 80% of the surface area of the culture plate, usually every 4–6 days (see [Figure 1](#)). [Troubleshooting 2](#) and [3](#).
 - a. Aspirate the Complete E8 Medium from each well of 6-well plate.
 - b. Wash with 2 mL of DPBS 1 $\times$ per well.
 - c. Add 1 mL of 0.5 mM EDTA per well and incubate for 4–6 min at 20°C–22°C. During incubation with EDTA, observe the hPSC colonies under the microscope. When the cells in the colonies start to round up and detach, they are ready to be removed from the well.

△ CRITICAL: Avoid longer EDTA exposure. Excessive cell dissociation may result in low cell viability.

- d. Aspirate the EDTA solution from the wells without disrupting the hPSC colonies. Then, dissociate the colonies by flushing them with 1 mL of Complete E8 Medium (2–3 times) to break up the colonies into small cell clusters.

△ **CRITICAL:** Do not pipette the cell clusters more than 3 times, because hPSC are sensitive to mechanical stress. Excessive mechanical disruption would result in low cell viability.

- e. Re-suspend the cell clusters in Complete E8 Medium and plate 2 mL of the cell suspension per well in a new VTN-N coated 6-well plate. Use a split ratio ranging from 1:3 to 1:4 for the first 2 passages post-thaw and from 1:6 to 1:10 for subsequent passages.

△ **CRITICAL:** Do not add ROCK inhibitor to the medium during hPSC passage.

Note: The optimal split ratio and dilution ratio during a split may vary among different hPSC lines and will need to be adjusted to allow growth of the cell colonies for 4–6 days until reaching 80% confluency.

- f. Incubate the 6-well plate containing hPSC at 37°C and 5% CO₂. Refresh with 2 mL of Complete E8 Medium per well the day after cell plating. Change medium every other day.

14. Prepare frozen stocks of hPSC.

- a. Prepare Freezing Medium containing 10% DMSO by mixing 9 mL of Complete E8 Medium with 1 mL of DMSO in a sterile 15 mL conical tube.
- b. When hPSC colonies cover 80% of the surface area of the culture plate, dissociate them from each well of 6-well plate into small cell clusters using 0.5 mM EDTA as detailed in steps 13a–d.

Note: Usually freeze 1 well of 6-well plate of 80% confluent hPSC per cryovial.

- c. Centrifuge the cell suspension at 300 × g for 5 min. Remove the supernatant. For each well of dissociated hPSC, use 1 mL of ice-cold Freezing Medium to gently re-suspend the cell pellet.
- d. Transfer 1 mL of the cell suspension using a p1000 micropipette into each labelled cryovial. Immediately place the cryovials with the cells into a Mr Frosty™ Freezing Container and store at –80°C for 24 h.
- e. The day after, transfer the frozen cryovials from –80°C freezer to a nitrogen tank.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human/mouse/rat/hamster angiotensin-converting enzyme 2 (ACE2) antibody, Source: Polyclonal Goat IgG, Working dilution: 1:20	Bio-Techne R&D Systems	Cat# AF933; RRID: AB_355722
SARS-CoV/SARS-CoV-2 nucleocapsid antibody for virus nuclear protein (NP) detection, Source: Monoclonal Mouse IgG1 Clone #05, Working dilution: 1:1000 (for immunofluorescence in Vero-E6 cells), 1:500 (for immunofluorescence in kidney organoids)	Abyntek Biopharma	Cat# 40143-MM05; RRID: AB_2827977
IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody, Working dilution: 1:5000	Licor	Cat# 926-32210; RRID: AB_621842
Alexa Fluor® 488 AffiniPure Donkey Anti-Goat IgG (H+L), Working dilution: 1:200	Jackson ImmunoResearch	Cat# 705-545-147; RRID: AB_2336933
Cy™3 AffiniPure Donkey Anti-Mouse IgG (H+L), Working dilution: 1:200	Jackson ImmunoResearch	Cat# 715-165-151; RRID: AB_2315777
Streptavidin, DyLight™ 649 Conjugated, Working dilution: 1:40	Vector Labs	Cat# SA-5649; RRID: AB_2336421
Bacterial and virus strains		
SARS-CoV-2, GENBANK: MT093571	Isolated from patient; Monteil et al. ² Garreta et al. ¹	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Lotus Tetragonolobus Lectin (LTL), biotinylated, Working dilution: 1:200	Vector Laboratories	Cat# B-1325; RRID: AB_2336558
Draq5	Thermo Fisher	Cat# 62254
4',6-Diamidino-2-phenylindole (DAPI)	Thermo Fisher	Cat# D1306
Essential 8 (E8) Medium consisting of Essential 8™ Basal Medium and Essential 8™ Supplement (50×)	Thermo Fisher	Cat# A1517001
Vitronectin (VTN-N), Recombinant Human Protein, Truncated	Thermo Fisher	Cat# A14700
Trypan Blue stain (0.4%)	Thermo Fisher	Cat# 15250061
Ethylenediaminetetraacetic acid (EDTA) solution	Thermo Fisher	Cat# 15575038
ACCUMAX™ Cell detachment solution	Stem Cell Technologies	Cat# 07921
0.25% Trypsin-EDTA solution (1×)	Thermo Fisher	Cat# 25200056
TrypLE™ Express	Thermo Fisher	Cat# 12604021
Advanced RPMI 1640	Thermo Fisher	Cat# 12633020
Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA) (100×)	Thermo Fisher	Cat# 11140035
HEPES (1 M) buffer solution	Thermo Fisher	Cat# 15630080
GlutaMAX™ Supplement (200 mM)	Thermo Fisher	Cat# 35050038
Penicillin/streptomycin (P/S) (10,000 U/mL)	Thermo Fisher	Cat# 15140122
Fetal bovine serum (FBS)	Thermo Fisher	Cat# 10270106
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher	Cat# 11995065
DMEM, glucose-free	Thermo Fisher	Cat# 11966025
Minimum Essential Medium – Eagle with Earle's BSS (EMEM) (2×)	Lonza	Cat# BE12-668F
D-Glucose	Merck	Cat# G7021
D-Mannitol	Merck	Cat# M9647
CHIR99021 (CHIR)	Merck	Cat# SML1046; CAS: 2 52917-06-9
Recombinant human FGF9	PeproTech	Cat# 100-23
Heparin	Merck	Cat# H3149; CAS: 9041-08-1
Activin A	R&D Systems	Cat# 338-AC-050
Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (1×)	Thermo Fisher	Cat# 14190144
Distilled water, cell culture grade	Thermo Fisher	Cat# 15230089
Phosphate buffered saline (PBS) pH 7.4 (1×)	Thermo Fisher	Cat# 1001015
Fluoromount-G	Southern Biotech	Cat# 0100-01
RapiClear® 1.47	Sunjin Lab	Cat# RC147001
Triton X-100	Sigma	Cat# T8787
Dimethyl sulfoxide (DMSO)	Merck	Cat# D2650; CAS: 67-68-5
Formaldehyde solution (min. 37%)	Sigma-Aldrich	Cat# 1039991000
Paraformaldehyde (PFA)	Anane	Cat# sc-281692
Donkey serum (DS)	Sigma-Aldrich	Cat# S30-100ML
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# A4503
Trizma base	Sigma-Aldrich	Cat# T6791
Sodium chloride (NaCl)	Sigma-Aldrich	Cat# S7653
Sodium azide	Sigma-Aldrich	Cat# 71290
Low gelling temperature type VII Agarose	Sigma-Aldrich	Cat# A4018
SeaKem ME Agarose	Lonza	Cat# 50014
Crystal Violet	Thermo Fisher	Cat# C581-100
Ethanol absolute (99.8%)	PanReac AppliChem	Cat# PANR.141086.1211; CAS: 64-17-5
Hydrochloric Acid (HCl), 1 mol/L (1 N) volumetric solution	PanReac AppliChem	Cat# 181021.1211 CAS: 7647-01-0
Critical commercial assays		
Streptavidin/Biotin Blocking Kit	Vector Laboratories	Cat# SP-2002
Intercept® (PBS) Blocking Buffer	Licor	Cat# 927-70001
Experimental models: Cell lines		
ES[4] Human Embryonic Stem Cell line	The National Bank of Stem Cells (ISCIII, Madrid)	https://www.isciii.es/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CBiPS1sv-4F-40 Human Induced Pluripotent Stem Cell line	The National Bank of Stem Cells (ISCIII, Madrid)	https://www.isciii.es/
Vero-E6	ATCC	Cat# ATCC CRL-1586
Software and algorithms		
Empiria Studio® Software	Licor	N/A
Fiji ImageJ2 version 2.3.0	NIH	https://imagej.net/software/fiji/
Other		
Countess™ Cell Counting Chamber Slides	Thermo Fisher	Cat# C10228
Falcon® 15 mL Polystyrene Centrifuge Tube, Conical Bottom, with Dome Seal Screw Cap, Sterile	Corning	Cat# 352099
Falcon® 50 mL High Clarity PP Centrifuge Tube, Conical Bottom, Sterile	Corning	Cat# 352070
Screw Cap Micro Tube, working volume: 2 mL, skirted conical base, Sterile	Sarstedt	Cat# 72.694.005
Eppendorf™ Safe-Lock Tubes, 1.5 mL	Fisher Scientific	Cat# 10509691
Mr Frosty™ Freezing Container	Thermo Fisher	Cat# 5100-0001
Fisherbrand™ Internally Threaded Cryogenic Storage Vials, 2 mL, Sterile	Fisher Scientific	Cat# 11321675
Merck Millex™-GP Sterile Syringe Filter Units with PES Membrane, 0.22 µm	Fisher Scientific	Cat# 16437555
Nunc™ EasYFlasks™ TC-treated with Filter Cap, 75-cm ² flask	Thermo Fisher	Cat# 156499
Nunc™ 96-Well Polystyrene Conical Bottom (V-bottom) MicroWell™ Plates	Thermo Fisher	Cat# 249935
96 Well Black/Clear Bottom Plate, TC Surface	Thermo Fisher	Cat# 165305
96 Well Plate, Sphera Low-Attachment Surface	Thermo Fisher	Cat# 174927
200 µL Graduated Tip/Wide Orifice, Racked	Ecogen	Cat# E1011-8400
Histology plastic molds	Laboquimia	Cat# 20447200820
Charged glass slides SUPERFROST Plus	VWR	Cat# 631-9483
Menzel-Gläser Coverslip 24 × 50 mm #1.5, Rectangular coverslips, 24 × 50 mm, #1.5	VWR	Cat# 631-9430
iSpacer®	Sunjin Lab	Cat# IS211

MATERIALS AND EQUIPMENT

Preparation of medium for Vero-E6 cell culture and infection with SARS-CoV-2

Complete Growth Medium

Reagent	Final concentration	Volume (mL)
DMEM (High Glucose)	N/A	435
P/S (10.000 U/mL)	1%	5
MEM NEAA 100x	1%	5
FBS	10%	50
HEPES (1 M)	10 mM	5
Total	N/A	500

Store at 4°C, use within 6 weeks, bring to 37°C prior to use.

Preparation of reagents and solutions for the plaque assay and TCID50

Complete Infection Medium

Reagent	Final concentration	Volume (mL)
DMEM (High Glucose)	N/A	485
P/S (10.000 U/mL)	1%	5
FBS	2%	10
Total	N/A	500

Store at 4°C, use within 6 weeks, bring to 37°C prior to use.

Agarose solution (1%)

Reagent	Final concentration	Amount
SeaKem ME Agarose	1%	1 g
Deionized distilled (Milli-Q) water	N/A	100 mL
Total	1%	100 mL

Dissolve 1g of Agarose in 100 mL of boiling Milli-Q water. Autoclave. Keep at 20°C–22°C.

Before to use, melt the agarose using a microwave and keep at 55°C in a water-bath until use.

EMEM/14% FBS Medium

Reagent	Final concentration	Volume (mL)
EMEM 2x	N/A	42
FBS	14%	7
GlutaMAX (200 mM)	2%	1
Total	N/A	50

Store at 4°C, use within 6 weeks, bring to 20°C–22°C prior to use.

Plaque Assay Overlay Medium

Reagent	Final concentration	Volume (mL)
EMEM/14% FBS Medium	N/A	50
Agarose solution (1%)	0.5%	50
Total	N/A	100

Equilibrate the EMEM+14% FBS at 20°C–22°C. Prepare the plaque assay overlay medium just before adding the overlay on cells by mixing at 1:1 dilution ratio EMEM+14%FBS (at 20°C–22°C) and 1% agarose solution (at 55°C). Use immediately to overlay the cell monolayers.

Formaldehyde solution (25%)

Reagent	Final concentration	Volume (mL)
Formaldehyde (37%)	25%	33.8
PBS 1x	N/A	16.2
Total	25%	50 mL

Store at 4°C, use within 1 week. Formaldehyde solutions must be disposed of safely and in accordance with the local regulations.

Crystal Violet staining solution (1% w/v)

Reagent	Final concentration	Amount
Crystal violet	1%	1 g
Absolute Ethanol	20%	20 mL
Formaldehyde (37%)	25%	25 mL
PBS 1x	N/A	55 mL
Total	1%	100 mL

Store at 20°C–22°C protected from light, use within 4 months. Mix before each use.

Preparation of medium for hPSC maintenance

Complete E8 Medium

Reagent	Final concentration	Volume (mL)
E8 Basal Medium	N/A	485
E8 Supplement 50x	N/A	10
P/S (10,000 U/mL)	1%	5
Total	N/A	500

Store at 4°C, use within 2 weeks. Before use, warm only the required amount of medium at 20°C–22°C. Do not warm the medium at 37°C. Protect from direct light.

Stock preparation of medium components for kidney organoid generation

Stock solution of CHIR

Reagent	Final concentration (mM)	Amount
CHIR	12	5 mg
DMSO	N/A	896 μ L
Total	12	896 μL

Make 50 μ L working aliquots and store at -20°C , use within 6 months.

Stock solution of Activin A (50 μ g/mL)

Reagent	Final concentration (μ g/mL)	Amount
Activin A	50	50 μ g
Sterile HCl (4 mM)	N/A	1,000 μ L
Total	50	1,000 μL

Make 20–50 μ L working aliquots and store at -20°C , use within 6 months. Avoid repeated freezing and thawing cycles.

Stock solution of Recombinant Human FGF9 (50 μ g/mL)

Reagent	Final concentration (μ g/mL)	Amount
FGF9	50	500 μ g
Distilled Water, Cell culture grade	N/A	10,000 μ L
Total	50	10,000 μL

Make 100 μ L working aliquots and store at -20°C , use within 6 months. Avoid repeated freezing and thawing cycles.

Stock solution of Heparin (50 mg/mL)

Reagent	Final concentration (mg/mL)	Amount
Heparin	50	50 mg
Distilled Water, Cell culture grade	N/A	1,000 μ L
Total	50	1,000 μL

Make 50 μ L aliquots and store at 4°C , use within 2 years. For regular use, prepare an intermediate heparin stock solution at 5 mg/mL by diluting the 50 mg/mL stock at 1:10 ratio.

Preparation of medium for kidney organoid generation

Complete Advanced RPMI 1640 Medium

Reagent	Final concentration (%)	Volume (mL)
Advanced RPMI 1640 Basal Medium	N/A	49
P/S (10,000 U/mL)	1	0.5
GlutaMAX (200 mM)	1	0.5
Total	N/A	50

Store at 4°C , use within 4 weeks, bring to 20°C – 22°C prior to use.

Posterior Primitive Streak Induction Medium

Reagent	Final concentration	Volume
Complete Advanced RPMI 1640 Medium	N/A	15 mL
CHIR (12 mM)	8 μ M	10 μ L
Total	N/A	15 mL

Prepare fresh, bring to 20°C – 22°C prior to use.

Intermediate Mesoderm Induction Medium

Reagent	Final concentration	Volume
Complete Advanced RPMI 1640 Medium	N/A	15 mL
FGF9 (50 µg/mL)	200 ng/mL	60 µL
Activin A (50 µg/mL)	10 ng/mL	3 µL
Heparin (5 mg/mL)	1 µg/mL	3 µL
Total	N/A	15 mL

Prepare fresh, bring to 20°C–22°C prior to use. For heparin, use an intermediate stock solution at 5 mg/mL by diluting the 50 mg/mL stock at 1:10 ratio.

CHIR-Pulse Organoid Induction Medium

Reagent	Final concentration	Volume
Complete Advanced RPMI 1640 Medium	N/A	15 mL
FGF9 (50 µg/mL)	200 ng/mL	60 µL
Heparin (5 mg/mL)	1 µg/mL	3 µL
CHIR (12 mM)	5 µM	6.25 µL
Total	N/A	15 mL

Prepare fresh, bring to 20°C–22°C prior to use. For heparin, use an intermediate stock solution at 5 mg/mL by diluting the 50 mg/mL stock at 1:10 ratio.

Kidney Organoid Differentiation Medium

Reagent	Final concentration	Volume
Complete Advanced RPMI 1640 Medium	N/A	40 mL
FGF9 (50 µg/mL)	200 ng/mL	160 µL
Heparin (5 mg/mL)	1 µg/mL	8 µL
Total	N/A	40 mL

Prepare fresh, bring to 20°C–22°C prior to use. For heparin, use an intermediate stock solution at 5 mg/mL by diluting the 50 mg/mL stock at 1:10 ratio.

Stock preparation of medium components and complete medium for high oscillatory glucose treatment of kidney organoids

Stock solution of D-Glucose (1 M)

Reagent	Final concentration	Amount
D-Glucose	1 M	18.0 g
Distilled Water, Cell culture grade	N/A	100 mL
Total	1 M	100 mL

Mix 18 g of glucose with 80 mL of cell culture grade distilled water using magnetic stirring until complete dissolution. Then, bring to a total volume of 100 mL with cell culture grade distilled water. Filter the solution using a 0.22 µm pore size syringe filter and store at 4°C, use within 2 months. Do not autoclave glucose solutions.

Stock solution of D-Mannitol (1 M)

Reagent	Final concentration	Amount
D-Mannitol	1 M	18.2 g
Distilled Water, Cell culture grade	N/A	100 mL
Total	1 M	100 mL

Mix 18.2 g of mannitol with 80 mL of cell culture grade distilled water using magnetic stirring until complete dissolution. Then, bring to a total volume of 100 mL with cell culture grade distilled water. Filter the solution using a 0.22 µm pore size syringe filter. Store at 20°C–22°C to avoid crystal formation, use within 2 months.

Normoglycemic Medium (5 mM Glucose)

Reagent	Final concentration	Volume (mL)
DMEM (Glucose free)	N/A	477.5
P/S (10,000 U/mL)	1%	5
GlutaMAX (200 mM)	1%	5
D-Glucose (1 M)	5 mM	2.5
D-Mannitol (1 M)	20 mM	10
Total	N/A	500

Store at 4°C, use within 6 weeks, bring to 37°C prior to use.

Hyperglycemic Medium (25 mM Glucose)

Reagent	Final concentration	Volume (mL)
DMEM (glucose free)	N/A	477.5
P/S (10,000 U/mL)	1%	5
GlutaMAX (200 mM)	1%	5
D-Glucose (1 M)	25 mM	12.5
Total	N/A	500

Store at 4°C, use within 6 weeks, bring to 37°C prior to use.

Preparation of solutions for immunofluorescence analysis of kidney organoids

PFA fixative solution (4%)

Reagent	Final concentration	Volume (mL)
PFA (16%)	4%	12.5
PBS 1×	N/A	37.5
Total	4%	50

Store at 4°C, use within 1 week. PFA solutions must be disposed of safely and in accordance with the local regulations.

Tris-Buffered Saline (TBS) 10× (pH 7.6)

Reagent	Final concentration	Amount
Trizma base	200 mM	24 g
NaCl	1.5 M	88 g
Distilled (Milli-Q) water	N/A	900 mL
Total	N/A	1,000 mL

Dissolve 24 g of Trizma base and 88 gr NaCl in 900 mL of distilled (Milli-Q) water. Then adjust pH to 7.6 with hydrochloric acid (HCl), and complete with distilled (Milli-Q) water until 1,000 mL final volume. Store at 4°C, use within 6 months.

TBS 1× (pH 7.6)

Reagent	Final concentration	Volume (mL)
TBS 10×	N/A	100
Distilled (Milli-Q) water	N/A	900
Total	N/A	1,000

Store at 4°C, use within 6 months.

TBS 1× supplemented with 1% Triton X-100

Reagent	Final concentration	Volume (mL)
TBS 1×	N/A	100
Triton 100-X	1%	1
Total	N/A	100

Store at 4°C, use within 2 weeks.

TBS 1× supplemented with 0.5% Triton X-100

Reagent	Final concentration	Volume (mL)
TBS 1×	N/A	100
Triton 100-X	0.5%	0.5
Total	N/A	100

Store at 4°C, use within 2 weeks.

Blocking buffer for whole mount immunofluorescence

Reagent	Final concentration	Volume
TBS 1× supplemented with 1% Triton X-100	N/A	9.4 mL
Donkey serum	6%	600 µL
Total	N/A	10 mL

Filter the solution using a 0.22 µm pore size syringe filter and store at 4°C, use within 1 week.

Blocking buffer for immunofluorescence in paraffin sections

Reagent	Final concentration	Volume
TBS 1× supplemented with 1% Triton X-100	N/A	9.7 mL
Donkey Serum	3%	300 µL
Total	N/A	1,000

Filter the solution using a 0.22 µm pore size syringe filter and store at 4°C, use within 1 week.

TBS 1× supplemented with 1% Triton X-100 and 1% BSA

Reagent	Final concentration	Amount
TBS 1× supplemented with 1% Triton X-100	N/A	50 mL
BSA	1%	0.5 g
Total	N/A	50 mL

Filter the solution using a 0.22 µm pore size syringe filter and store at 4°C, use within 1 week.

TBS 1× supplemented with 0.5% Triton X-100 and 1% BSA

Reagent	Final concentration	Amount
TBS 1× supplemented with 0.5% Triton X-100	N/A	50 mL
BSA	1%	0.5 g
Total	N/A	50 mL

Filter the solution using a 0.22 µm pore size syringe filter and store at 4°C, use within 1 week.

Primary antibody dilution ratios for whole mount or paraffin section immunofluorescence

Primary antibody or reagent	Dilution ratio
ACE2	1:20
NP	1:500
LTL	1:200

Dilute the primary antibodies at the indicated dilution ratios in TBS 1× supplemented with 1% Triton X-100 and 1% BSA for whole mount immunofluorescence or in TBS 1× supplemented with 0.5% triton X-100 and 1% BSA for organoid paraffin sections. Prepare fresh.

Secondary antibody dilution ratios for whole mount or paraffin section immunofluorescence

Secondary antibody or reagent	Dilution ratio
Anti-Goat-AF488	1:200
Anti-mouse-Cy3	1:200
Streptavidin- Dylight 649	1:40

Dilute the secondary antibodies at the indicated dilution ratios in TBS 1× supplemented with 1% Triton X-100 and 1% BSA for whole mount immunofluorescence or in TBS 1× supplemented with 0.5% Triton X-100 and 1% BSA for organoid paraffin sections. Prepare fresh.

Stock of DAPI (5 mg/mL)

Reagent	Final concentration	Amount
DAPI	5 mg/mL (14.3 mM)	10 mg
Distilled (Milli-Q) water	N/A	2 mL
Total	5 mg/mL (14.3 mM)	2 mL

Make 20–50 µL aliquots and store at –20°C in the dark, use within 6 months. Avoid repeated freezing and thawing cycles.

DAPI working solution (1:5000)

Reagent	Final concentration	Volume
DAPI (5 mg/mL)	1 µg/mL	2 µL
TBS 1×	N/A	10 mL
Total	N/A	10 mL

Prepare fresh. DAPI solutions must be disposed of safely and in accordance with the local regulations.

STEP-BY-STEP METHOD DETAILS

Preparation of SARS-CoV-2 virus stocks

⌚ **Timing:** 4 days

This step allows the production of virus stock by amplification of a defined SARS-CoV-2 virus strain in Vero-E6 Cells. The infectivity of virus stock is assessed by 2 different means including plaque assay (next section, beginning at step 7) or by tissue culture infectious dose-50 (TCID₅₀) assay (beginning at step 14).

1. Grow Vero-E6 Cells until 90% confluency in two 75-cm² flasks.
 - a. 24 h before virus inoculation, plate Vero-E6 Cells at $6.5\text{--}9.5 \times 10^4$ cells/cm² in ten 75-cm² flasks in Complete Growth Medium. Each 75-cm² flask will produce 15 mL of virus stock.
 - b. Plate 2 additional flasks, 1 for mock-infected control and the other one for cell counting.

Note: It is desirable to produce large amount of virus at a time to have consistency between experimental replicates. Usually, 10 flasks of Vero-E6 Cells for SARS-CoV-2 infection can be prepared. Vero-E6 Cells need to be 85%–90% confluent for virus inoculation and propagation.

- c. Incubate the flasks at 37°C and 5% CO₂.

⚠ **CRITICAL:** Restart the culture with a new early passage of Vero-E6 Cells if changes in morphology or if virus titers have decreased to below 10⁵ plaque-forming units (PFU)/mL.

⚠ **CRITICAL:** SARS-CoV-2 should not be passaged more than 3 times in Vero-E6 Cells to avoid viral genome mutations that may decrease or change its infectivity.

2. The day after seeding, count the cells and calculate the volume of virus inoculum to have a multiplicity of infection (MOI) of 0.01 PFU per cell using the following formula:

Volume of virus needed in mL = (Number of cells in the flask) × (desired MOI) / (virus inoculum titer in PFU/mL).

Note: A 90% confluent 75-cm² flask contains approximately $6.5\text{--}8 \times 10^6$ cells. For a virus titer of 10^7 PFU/mL, the volume of virus needed to inoculate 1 75-cm² flask of Vero-E6 Cells would be: $(6.5 \times 10^6) \times (0.01) / 10^7 = 0.0065 \text{ mL} = 6.5 \mu\text{L}$ per flask.

△ CRITICAL: To produce SARS-CoV-2 virus stocks, it is important to inoculate the virus at low MOI (MOI=0.01 per cell) to ensure that each cell is productively infected while preventing the formation of defective viral particles.

△ CRITICAL: Steps 1–2 can be performed in BSL-1 or BSL-2 containment. From this step on, all procedures (steps 3–6) must occur under BSL-3 containment.

3. Thaw a vial of SARS-CoV-2 seed stock at 20°C–22°C.
4. Dilute the necessary volume of virus (calculated above) in DMEM supplemented with 2% FBS (Complete Infection Medium).
5. Inoculate 90% confluent Vero-E6 Cell monolayers with virus.
 - a. Remove the medium from the 75-cm² flasks containing 90% confluent Vero-E6 Cells.
 - b. Wash once with 3 mL of PBS 1 × per flask.
 - c. Add 3 mL of Complete Infection Medium containing the virus (from step 4) to each 75-cm² flask. For mock-infected control, add 3 mL of fresh Complete Infection Medium.
 - d. Incubate flasks at 37°C and 5% CO₂ for 1 h.
 - e. After 1 h, add 7 mL of Complete Growth Medium to each flask.
 - f. Monitor the flasks daily for 48–72 h for the appearance of cytopathic effect (CPE) using an inverted microscope (see [Figure 2A](#)). Compare with mock-infected flask as negative control.
6. When 90% CPE is observed, harvest the virus supernatant from infected Vero-E6 Cell monolayers.
 - a. Collect the virus supernatant into sterile 15 mL conical tubes. Discard the mock-infected control.
 - i. Dispose flasks, remaining cells and pipettes as infectious waste.
 - b. Centrifuge the tubes at $450 \times g$ for 5 min at 4°C to pellet the cell debris.
 - c. Prepare 200–500 μL aliquots of the supernatant in labelled 2 mL screw-tubes.
 - d. Immediately store the aliquots of virus stock at –80°C.

Note: The volume of virus stock aliquots will need to be adjusted to avoid repeated freezing and thawing cycles. This will prevent decrease of virus stock infectivity.

Titration of SARS-CoV-2 virus stock by plaque assay

⌚ Timing: 4 days

This step is required to determine the virus stock titer by plaque assay, which measures infectious SARS-CoV-2 particles by quantifying the plaques generated in Vero-E6 Cell cultures after infection with serial dilutions of the virus stock using a viscous medium overlay. The viscous overlay is used to restrict the spreading of progeny virions to the initially infected foci of cells. In this manner, each infectious particle will generate a single area of cellular death, so called PFU. After incubation, cell monolayers are fixed and stained to visualize the plaques using crystal violet, which stains cells in purple while plaques remain clear. Plaques are then counted to assess the virus titer in PFU per mL (PFU/mL).

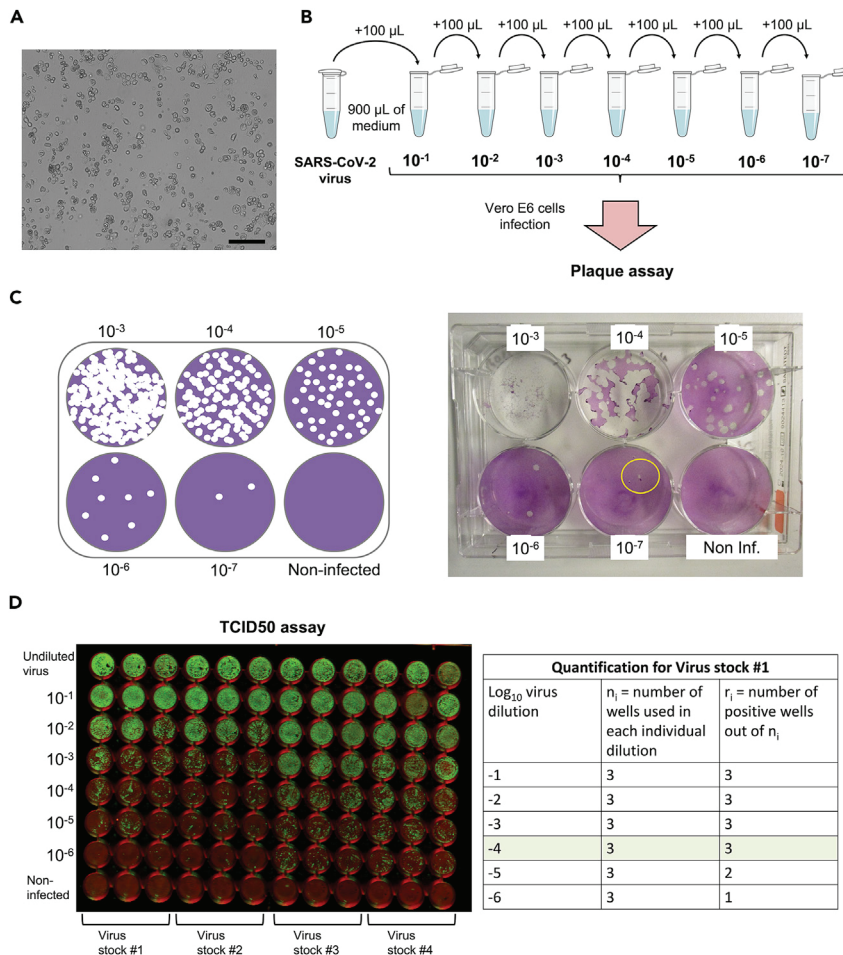


Figure 2. Propagation and titration of SARS-CoV-2 virus in Vero-E6 Cells

(A) Representative bright field image of Vero-E6 Cell monolayer with apparent CPE 72 h after SARS-CoV-2 virus inoculation. Scale bar, 100 μ m.

(B) Schematics of the virus serial dilutions preparation.

(C) Schematics of a 6-well plate used for the plaque assay after staining with the crystal violet solution (left) and the corresponding image of a real plaque assay plate (right) showing the formation of the plaques. Note that the well corresponding to the 10^{-7} virus dilution does not show plaque formation but presents a scratch (indicated with a yellow circle) that has been accidentally performed when removing the agarose layer with the flat spoon and can be confounded with a plaque.

(D) Representative fluorescent image of a typical 96-well plate prepared for virus titer assessment by the TCID₅₀ assay (left). The table (right) shows a representative quantification of the number of positive infected wells that are used to calculate the virus titer by the Spearman-Kärber method.

7. Prepare Vero-E6 Cell monolayers for plaque assay.

- Plate 8×10^5 Vero-E6 Cells in 3 mL of Complete Growth Medium per well of 6-well plate.

Note: One 6-well plate is prepared per virus stock to be tested.

- Incubate the plate for 12–16 h at 37°C and 5% CO₂.

- After 12–16 h incubation, check under the microscope that cells formed a confluent compact monolayer in each well.

△ CRITICAL: Step 7 can be performed in BSL-1 or BSL-2 containment. From this step on, all procedures (steps 8–13) must occur under BSL-3 containment.

8. Heat the 1% Agarose solution in a microwave and keep the bottle in a water-bath at 55°C.
9. Prepare EMEM/14% FBS Medium and equilibrate it at 20°C–22°C.
10. Dilute the virus stock (see [Figure 2B](#)).
 - a. Prepare 10-fold serial dilutions of the virus sample between 10^{-1} to 10^{-7} .
 - i. For each virus sample to be titrated, take seven 1.5 mL Eppendorf tubes and label them with each dilution (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7}).
 - ii. Add 900 μ L of Complete Infection Medium to each tube.
 - iii. Add 100 μ L of the virus sample to be titrated to the first tube (10^{-1}) to make the first dilution.
 - iv. Mix by vortexing the tube. This is your 10^{-1} virus dilution.
 - v. Continue with serial dilutions by taking 100 μ L of the first virus dilution (10^{-1}) and transferring it to the second tube (10^{-2}).
 - vi. Mix by vortexing the tube. This is now your 10^{-2} virus dilution.
 - vii. For subsequent virus dilutions repeat steps v–vi by taking 100 μ L of the newly created virus dilution and adding it to the next Eppendorf tube until you have reached a 10^{-7} dilution.

△ CRITICAL: Directly discard the tips without pipetting between each dilution.

11. Infect Vero-E6 Cell monolayers prepared in step 7 with the serial virus dilutions.
 - a. Label each well of 6-well plate with the corresponding dilution ranging from 10^{-3} to 10^{-7} and 1 well for non-infected control.
 - b. Remove the existent Complete Growth Medium from the Vero-E6 Cell monolayers.
 - c. Wash once with 1 mL of PBS 1× per well.
 - d. After vortexing the tube, transfer 500 μ L of the 10^{-7} virus dilution to one well of the labelled 6-well plate.
 - e. Repeat this step for 10^{-6} to 10^{-3} dilutions.

Note: It is recommendable to start with the most diluted sample so the same pipette tip can be then used for subsequent pipetting from the most diluted to the less diluted samples.

- f. Incubate the plates at 37°C and 5% CO₂ for 1 h to allow virus to attach.

△ CRITICAL: Redistribute the supernatant by rocking the plates carefully every 15 min to prevent cell monolayers from drying out.

- g. During the incubation time, calculate the volume of Plaque Assay Overlay Medium needed, counting 3 mL per well of 6-well plate. Include 3 mL extra as a control of polymerization.

Note: For example, for a 6-well plate, 6 × 3 mL + 3 mL are needed, i.e 21 mL of plaque assay overlay medium.

- h. After 1 h incubation, remove the viral inoculums from each well and close the plate.

△ CRITICAL: Change tips between each well.

- i. Prepare the Plaque Assay Overlay Medium in a 50 mL conical tube, by quickly mixing 1 volume of 1% Agarose solution (equilibrated at 55°C, see step 8) and 1 volume of EMEM/14% FBS Medium (equilibrated at 20°C–22°C, see step 9) (1:1 ratio).

△ CRITICAL: The overlay medium has to be prepared just before being distributed in the plate because agarose begins to polymerize rapidly after mixing with the EMEM/14% FBS Medium.

- j. Add 3 mL of Plaque Assay Overlay Medium to each well of 6-well plate.

△ CRITICAL: Let the overlay polymerize in the plate under the hood. To check the polymerization status, check the status of the leftover in the 50 mL tube.

- k. Incubate the plates at 37°C and 5% CO₂ for 3 days to allow plaque formation.

12. Visualize plaques by Crystal Violet staining 3 days post-infection (see [Figure 2C](#)). [Troubleshooting 4](#) and [5](#).

Note: Crystal Violet stains proteins and DNA within intact cells. In this manner, areas of infected dead cells will appear as clear spots on the purple cell monolayer.

- a. Add 1 mL of 25% Formaldehyde solution per well on top of the agarose to allow fixation of the cell monolayers.
- b. Incubate for 30 min at 20°C–22°C.

Note: The fixation step with 25% formaldehyde solution allows for virus inactivation.

- c. Carefully remove the agarose layer using a flat spatula. Dispose as infectious waste.

△ CRITICAL: Do not touch the cell monolayer with the spatula to avoid scratching the monolayer.

- d. Add 1 mL of 1% Crystal Violet staining solution per well of the 6-well plate.
- e. Incubate for 10 min at 20°C–22°C.
- f. Remove the Crystal Violet solution from the wells and wash 3 times with 3 mL of tap water per well.

△ CRITICAL: Properly discard formaldehyde and crystal violet solutions as toxic waste, according to institutional rules.

- g. Leave the plate inverted to air-dry the stained monolayers.

▮▮ Pause point: At this point, air-dried stained plates can be stored at 20°C–22°C for further quantification.

13. Enumerate plaques and calculate the virus titer. [Troubleshooting 6](#) and [7](#).

- a. Count the plaques in each well at each virus dilution.

Note: Discount wells with fewer than 5 or greater than 100 plaques. Take note of plaque size and morphology. The negative control should present a uniform, purple-stained monolayer and is used as a reference control.

- b. Identify the first dilution in which a maximum of 50 plaques can be counted. Determine the viral titer of the virus using the following formula:

Plaque forming units (PFU)/mL = [average No. of plaques] / [Dilution factor × Volume of inoculum added in mL].

Note: For instance, if you counted 50 plaques in the well corresponding to 10⁻⁵ dilution in which you have added 0.5 mL of diluted virus inoculum, the titer of the virus stock would be 50 / (10⁻⁵ × 0.5) = 10⁷ PFU/mL.

Note: We recommend to run the titration in duplicate or to repeat the titration step using another tube from the same viral stock.

Titration of SARS-CoV-2 virus stock by TCID50 assay

⌚ Timing: 3 days

As an alternative method to the plaque assay, this step details how to determine the virus stock titer by TCID50 assay.

14. Prepare Vero-E6 Cell monolayers in a 96-well Black/Clear Bottom plate (96-BCB plate).
 - a. Plate 30,000 Vero-E6 Cells in 100 μ L of Complete Growth Medium per well of a 96-BCB plate. Prepare enough wells to test each virus dilution in triplicate.

Note: One 96-BCB plate can be used to test 4 different virus stocks in triplicate (see [Figure 2D](#)).

- b. Incubate the plate 12–16 h at 37°C and 5% CO₂.
- c. After 12–16 h incubation, check under the microscope that cells have formed a confluent compact monolayer in each well.

⚠ CRITICAL: Step 14 can be performed in a BSL-1 or BSL-2 containment. Steps 15–21 must occur under BSL-3 containment.

15. Infect the Vero-E6 Cells prepared in step 14 with 10-fold serial virus dilutions.
 - a. Remove the 100 μ L of media from the first row of wells of the 96-BCB plate containing Vero-E6 Cells.
 - b. Add 110 μ L of non-diluted virus stock per well.
 - c. Use a multichannel pipette to remove 10 μ L of virus from the first row of wells and place it in the second row of wells. Gently pipette up and down to mix the virus with the media. This is now your 10⁻¹ virus dilution.
 - d. Repeat step 15c by removing 10 μ L of the virus dilution from the second row of wells (10⁻¹) and adding it to the third row of wells. This is now your 10⁻² virus dilution.
 - e. For subsequent virus dilutions repeat step 15c by removing 10 μ L of the newly created virus dilution and adding it to the next row of wells until you have reached a 10⁻⁶ dilution.
 - f. Incubate the 96-BCB plate for 24 h at 37°C and 5% CO₂.

⚠ CRITICAL: Change the pipette tips for each dilution and make sure to properly mix the contents of the well before diluting into the next well. Leave the last lane as a mock sample.

16. Fix the 96-BCB plate at 24 h post-infection.
 - a. Remove the virus-infected media.
 - b. Wash the wells 1 time with 200 μ L of PBS 1× per well.
 - c. Add 200 μ L of 4% Paraformaldehyde (PFA) to each well and incubate at 20°C–22°C for 20 min.
 - d. Remove the PFA and wash the wells 3 times with 200 μ L of PBS 1× per well.

Note: The fixation step with 4% PFA allows for virus inactivation.

⚠ CRITICAL: After the 20 min-fixation, the full plate should be submerged in 4% PFA solution and incubated for additional 20 min to ensure that any virus found in the plate is

inactivated. Once completely inactivated, next steps can be performed in the BSL-1/BSL-2 laboratory.

17. Perform immunolabelling of the fixed 96-BCB plate to detect virus infection (see [Figure 2D](#)).
 - a. Add 100 μ L of PBS 1 $\times$ supplemented with 0.05% Triton-X to each well. Incubate at 20°C–22°C for 15 min.
 - b. Remove the solution and add 100 μ L per well of the Intercept® (PBS) Blocking Buffer. Incubate at 20°C–22°C for 30 min.
 - c. Prepare the necessary primary antibody solution by diluting the primary antibody against the virus nuclear protein (NP) at 1:1000 dilution ratio in Intercept® (PBS) Blocking Buffer.
 - d. Incubate with the primary antibody solution as indicated below:
 - i. Remove the Intercept® (PBS) Blocking Buffer from the wells and add 100 μ L of the primary antibody solution to each well.
 - ii. Incubate at 20°C–22°C for 1 h.
 - e. Remove the primary antibody solution from the wells and wash 3 times with 100 μ L of PBS 1 $\times$ per well.
 - f. Prepare the necessary secondary antibody solution by diluting the anti-mouse IR800CW secondary antibody at 1:5000 dilution ratio in Intercept® (PBS) Blocking Buffer. Supplement this solution also with the nuclear dye Draq5 at 1:10000 dilution ratio.
 - g. Incubate with the secondary antibody solution as indicated below:
 - i. Add 100 μ L of the secondary antibody solution supplemented with the nuclear dye Draq5 to each well.
 - ii. Incubate at 20°C–22°C in the dark for 45 min.
 - h. Remove the secondary antibody solution and wash the wells 3 times with 100 μ L of PBS 1 $\times$ per well.
 - i. Add 200 μ L of PBS 1 $\times$ to each well and image the plate in an Odyssey® Imaging System (Odyssey® M, CLx or DLx models by Licor). Analyse with the Empiria Studio® Software.

Alternatives: An epifluorescence microscope can also be used instead of the Odyssey® Imaging System to image the immunolabelled plate. In this case, use PBS 1 $\times$ supplemented with 5% BSA as blocking buffer and perform the secondary antibody incubation using a secondary antibody conjugated to Alexa Fluor 488 at 1:1000 dilution ratio and the nuclear stain DAPI at 1:2000 dilution ratio in blocking buffer.

18. Determine the number of positive infected wells using the following method:
 - a. Determine the average intensity of mock-infected cells.
 - b. Determine the standard deviation of the intensity of mock-infected cells.
 - c. Multiple the standard deviation by 2.
 - d. Add the 2 $\times$ the standard deviation to the average mock value. All values above this are considered positive wells.

Note: For example – Average Intensity=180.2, standard deviation=23.4, 2 $\times$ standard deviation=46.8, therefore all wells which have an intensity over 180.2 + 46.8 = 227.1 are considered positive.

19. Use the Spearman-Kärber method to calculate the TCID₅₀/mL using the following formula:

$$\log_{10} 50\% \text{ end point dilution} = - (x_0 - d/2 + d \sum r_i/n_i).$$

x_0 = $\log_{10}$ of the reciprocal of the highest dilution (lowest concentration) at which all wells are positive.

d = $\log_{10}$ of the dilution factor (dilution factor= 10).

n_i = number of wells used in each individual dilution.

r_i = number of positive wells out of n_i .

Summation is started at dilution x_0 .

Note: For example, if the highest virus dilution at which all wells are positive is 10^{-4} , then $x_0 = 4$; $d = 1$; $\log_{10}$ of 50% endpoint dilution = $- [4 - \frac{1}{2} + 1 ((3+2+1)/3)] = -5.5$; Therefore, the 50% end point dilution = $10^{-5.5}$ and the titer of the virus = $10^{5.5}$ TCID₅₀/mL (see [Figure 2D](#)).

20. TCID₅₀/mL can be used to calculate the PFU/mL using the following conversion:

$$\text{PFU/mL} = \text{TCID}_{50} \times 0.69.$$

Note: For example, if TCID₅₀/mL = $10^{5.5}$, PFU/mL = $10^{5.5} \times 0.69 = 3.2 \times 10^5 \times 0.69 = 2.2 \times 10^5$ PFU/mL.

Generation of kidney organoids from human pluripotent stem cells

⌚ Timing: 18 days

The following steps describe the generation of kidney organoids from hPSC in a 96-well floating culture system.^{1,2} This protocol is an adaptation of our previous methodology describing the generation of kidney organoids using the transwell culture system³ and our previous published works using 96-well floating culture system.^{2,4} In the next section, day 16 kidney organoids generated in floating culture conditions are exposed to normoglycemic-like glucose culture regimes or high oscillatory glucose culture conditions to emulate a diabetic-like milieu (beginning at step 26).

21. Seeding of hPSC for differentiation (day -2 to day 0), see [Figure 3A](#). [Troubleshooting 8](#).

- a. 1 h prior cell dissociation, prepare one 24-well plate with VTN-N coating (as described in [before you begin](#) section). Use 0.3 mL of VTN-N solution per well of 24-well plate.

Note: A typical differentiation experiment is started from 1 VTN-N coated 24-well plate.

- b. Disaggregate 3 wells of 6-well plate with hPSC at 80% of confluency into small cell clusters, as it is performed for a normal hPSC passage (described in [before you begin](#) section).

Note: A typical differentiation experiment can be initiated from 3 wells of 6-well plate with hPSCs at 80% of confluency. The number of cells that can be obtained from 1 well of 6-well plate with hPSC colonies at 80% of confluency normally ranges from 2 to 3 million cells.

- c. Collect the resultant cell clusters in 6 mL of Complete E8 Medium in a sterile 15 mL conical tube.
- d. Count cells using a Countess Automated Cell Counter (see [before you begin](#) section step 10c).

Alternatives: A Neubauer cell counting chamber can be used to count viable cells under the microscope.

- e. Plate between $1-2 \times 10^5$ viable cells per well of VTN-N coated 24-well plate.

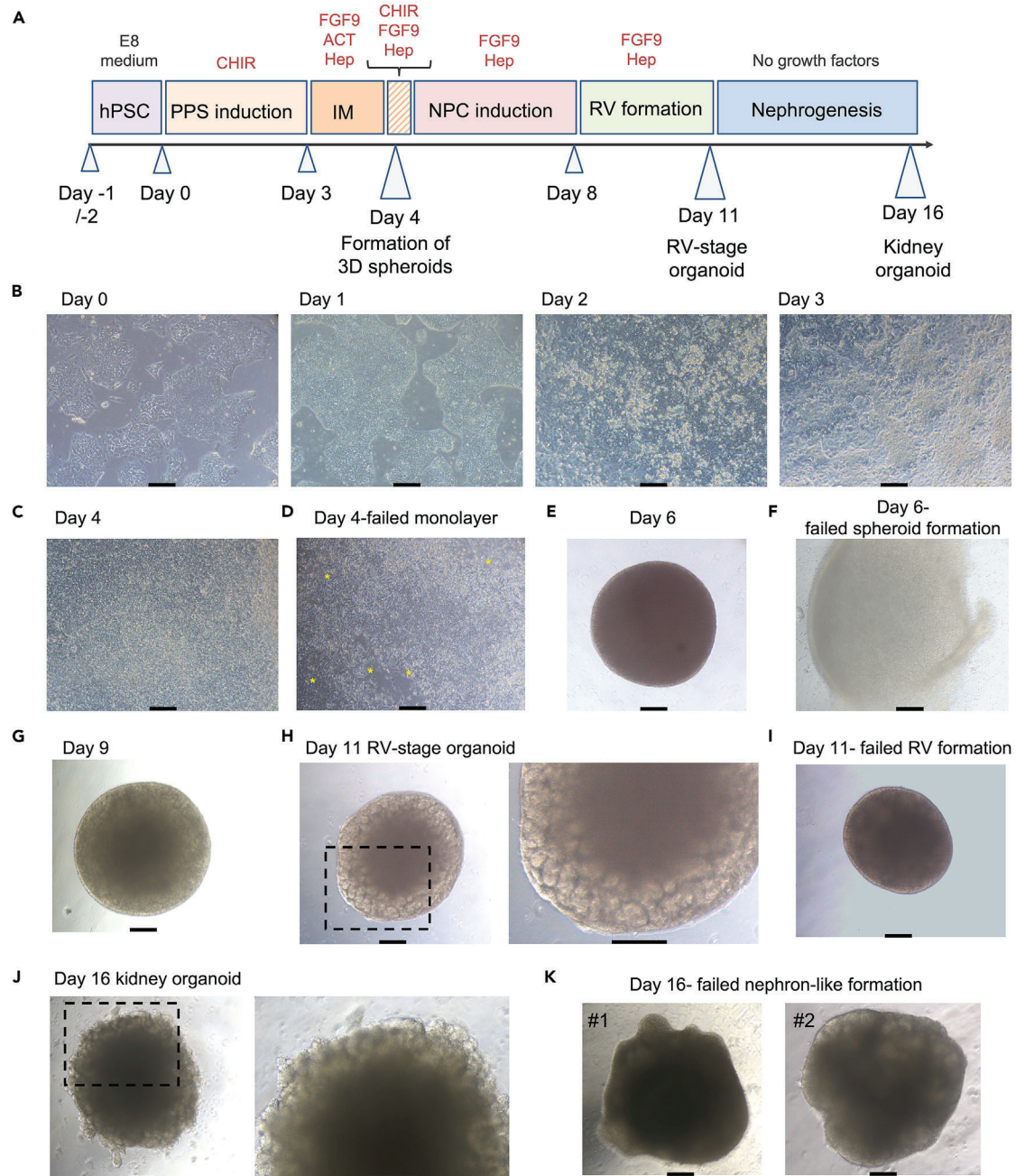


Figure 3. Generation of human kidney organoids from hPSC

(A) Timeline of kidney organoid generation from hPSCs. PPS: posterior primitive streak; IM: intermediate mesoderm; NPC: nephron progenitor cell; RV: renal vesicle; ACT: activin A; Hep: heparin.

(B) Representative bright field images showing morphological changes in the cell monolayer from day 0 to day 3 of differentiation.

(C) Representative bright field image of the cell monolayer at day 4 of differentiation showing a very compact and uniform appearance.

(D) Example of a failed formation of a day 4 cell monolayer. Yellow asterisks indicate areas of loose clusters of cells or empty areas.

(E) Representative bright field image of a day 6 spheroid (2 days after intermediate mesoderm-committed cell aggregation into spheroids). At this stage, cell spheroid is characterized by a well-compacted round morphology.

(F) Example of a failed cell aggregation in a day 6 spheroid. Note that edges are broken and disaggregated.

(G) Representative bright field image of a day 9 organoid that begin to show the formation of RVs in the organoid edges.

(H) Representative bright field image of a day 11 RV-stage organoid containing many RVs.

(I) Example of a failed formation of RVs in a day 11 kidney organoid.

(J) Representative bright field image of a day 16 kidney organoid containing multiple nephron-like structures.

(K) Examples of inefficient formation of nephron-like structures within day 16 kidney organoids. Scale bars in (B–K), 100 μ m.

△ **CRITICAL:** The starting cell density and colony distribution is crucial for an efficient differentiation and can vary among different hPSC lines. Usually, the optimal cell density ranges between $1\text{--}2 \times 10^5$ cells per well of 24-well plate and should be adjusted for every hPSC line.

- i. Calculate the volume of Complete E8 medium needed to achieve a cell density between $2\text{--}4 \times 10^5$ cells/mL. Re-suspend the cells and plate 0.5 mL of the cell suspension per well of VTN-N coated 24-well plate.
 - ii. After 24 h, refresh with Complete E8 Medium.
 - iii. Incubate at 37°C and 5% CO₂ for 24 h.
22. Induction of posterior primitive streak formation (day 0 to day 3), see [Figure 3A. Troubleshooting 9](#).
 - a. Gently aspirate the Complete E8 medium from each well of the 24-well plate prepared in step 21 without disrupting the hPSC colonies (day 0).
 - b. Add 0.5 mL of Complete Advanced RPMI 1640 Basal Medium supplemented with 8 μM CHIR (Posterior Primitive Streak Induction Medium) to each well. Incubate the plate at 37°C and 5% CO₂.
 - c. Replace the media with fresh Posterior Primitive Streak Induction Medium every 24 h for 3 consecutive days (see [Figure 3B](#)).
23. Induction of intermediate mesoderm formation (day 3 to day 4), see [Figure 3A. Troubleshooting 10](#).
 - a. Gently aspirate the Posterior Primitive Streak Induction Medium from each well of the 24-well plate avoiding to mechanically disrupt the cell monolayers (day 3).
 - b. Add 0.5 mL per well of Complete Advanced RPMI 1640 Basal Medium supplemented with 200 ng/mL FGF9, 1 μg/mL Heparin and 10 ng/mL Activin A (Intermediate Mesoderm Induction Medium). Incubate the plate at 37°C and 5% CO₂ for 24 h (see [Figures 3C and 3D](#)).

△ **CRITICAL:** It is key for the success of kidney organoid generation that the treatment with FGF9, Activin A and Heparin lasts exactly 24 h.

24. Generation of Kidney organoids (day 4 to day 16), see [Figure 3A](#).

△ **CRITICAL:** On day 4 of differentiation observe cells under a microscope and check for the presence of a tight homogeneous cell monolayer. If day 4 cell monolayers have not grown properly, showing large empty areas of cells, the organoid generation can be compromised.

- a. Gently aspirate the Intermediate Mesoderm Induction Medium from each well of the 24-well plate avoiding to mechanically disrupt the cell monolayers (day 4).
 - b. Add 0.5 mL of Complete Advanced RPMI 1640 basal medium supplemented with 5 μM CHIR, 200 ng/mL FGF9 and 1 μg/mL Heparin (CHIR-Pulse Organoid Induction Medium). Incubate the plate at 37°C and 5% CO₂ for 1 h.
 - c. After 1 h incubation, dissociate cell monolayers to generate a single cell suspension.

△ **CRITICAL:** At this point, cell monolayers are fragile and can break easily. Pay special attention during media changes and washing steps to avoid losing cells.

- i. Gently aspirate the supernatant without mechanically disrupting the cell monolayer.
 - ii. Rinse cell monolayers by adding 0.5 mL of PBS 1× per well of 24-well plate.
 - iii. Add 300 μL of TrypLE™ Express and incubate at 20°C–22°C for 1 min.
 - iv. Gently aspirate the TrypLE™ Express without mechanically disrupting the cell monolayer.

- v. Dissociate the cell monolayers by flushing 0.5 mL of Complete Advanced RPMI 1640 Basal Medium supplemented with 200 ng/mL FGF9 and 1 µg/mL Heparin (Kidney Organoid Differentiation Medium) to each well of 24-well plate.
- vi. Collect the resultant cell suspension in a 50 mL conical tube. Mix 10 µL of cell suspension and 10 µL of 0.4% Trypan Blue Stain, mix thoroughly and pipette 10 µL of the mixture into a Countess™ cell counting chamber slide. Count viable cells using a Countess Automated Cell Counter or a Neubauer chamber.

Note: The average number of day 4-intermediate mesoderm committed cells that are obtained out of 1 well of 24-well plate ranges from $1.5\text{--}3 \times 10^6$ cells.

- d. Plate 1×10^5 cells per well of Nunc™ 96-Well Polystyrene Conical Bottom (V-bottom) MicroWell™ plate.
 - i. Centrifuge the cell suspension at $300 \times g$ for 5 min and re-suspend the cells in Kidney Organoid Differentiation Medium at a cell density of 1×10^6 cells/mL.
 - ii. Use a 12-multichannel pipette to plate 100 µL of the resultant cell suspension per well of V-bottom MicroWell™ plate.
- e. Centrifuge V-bottom MicroWell™ plates at $300 \times g$ for 3 min to induce the formation of intermediate mesoderm-committed spheroids (1 single organoid per well will form). [Troubleshooting 11](#).
- f. Incubate the resultant organoids at 37°C and 5% CO₂ (see [Figures 3E and 3F](#)).
- g. Culture the organoids for 7 additional days (day 4 to day 11) in Kidney Organoid Differentiation Medium. Change medium every second day.
 - i. Use a 12-multichannel pipette to remove the media avoiding touching the organoids and to add 100 µL of fresh Kidney Organoid Differentiation Medium per well.

△ CRITICAL: When changing media of V-bottom MicroWell™ plates with organoids pay attention not to aspirate organoids by accident.

- ii. Incubate the plate at 37°C and 5% CO₂.
- h. On day 11 of differentiation, remove growth factors by culturing organoids in 100 µL per well of Complete Advanced RPMI 1640 Medium. Organoids with numerous renal vesicles (RV) should be visible by day 11 (see [Figures 3G–3I](#)). [Troubleshooting 12](#).

Note: Discard the experiment if RV have not appeared by day 12.

- i. Culture the developing kidney organoids for 5 additional days (day 11 to day 16) in Complete Advanced RPMI 1640 Medium. Change medium every second day.

Optional: From day 11 of differentiation, media changes can be performed every third day by adding 140 µL of Complete Advanced RPMI 1640 medium per well.

- 25. On day 16 of differentiation, kidney organoids show multiple nephron-like structures (see [Figures 3J and 3K](#)). [Troubleshooting 13](#).

Challenging kidney organoids with high oscillatory glucose to emulate a diabetic-like milieu *in vitro*

⌚ **Timing:** 7 days

This section details the steps required to treat kidney organoids (generated in steps 21–25 of previous section) with normoglycemic or high oscillatory glucose culture regimes.¹ Organoids cultured

under both experimental conditions are infected with SARS-CoV2 as described in the next section (step 28).

26. Maintain organoids in V-bottom MicroWell™ plate and expose them to normoglycemic (control) or high oscillatory glucose (diabetic) conditions for 7 days (from day 16 to day 23).
 - a. Use a 12-multichannel pipette to replace the media every day with 100 μ L per well of Normoglycemic or Hyperglycemic Medium. Incubate at 37°C and 5% CO₂.
 - b. Control organoids are cultured in Normoglycemic Medium only.
 - c. Diabetic organoids are exposed to Normoglycemic Medium on even days or Hyperglycemic Medium on odd days.

△ CRITICAL: When performing medium changes ensure to completely replace the media from each well to guarantee the proper exposure of kidney organoids to the desired glucose concentration. Pay attention not to aspirate organoids by accident.

27. After the 7 day-treatment, use control and diabetic kidney organoids for SARS-CoV-2 infection experiments (beginning at step 28).

Infection of kidney organoids with SARS-CoV-2

⌚ Timing: 2 days

This step describes the methodology to infect control or diabetic kidney organoids with SARS-CoV-2. Mock and SARS-CoV-2 infected kidney organoids are analysed to detect the viral RNA by qRT-PCR and the virus nuclear protein (NP) by immunofluorescence. For RNA extraction, qRT-PCR analysis and primer sequences, please refer to Garreta et al. (2022).¹ For immunofluorescence protocol see next section (beginning at step 31).

28. Calculate the amount of virus stock needed to infect a given number of kidney organoids with 10⁶ SARS-CoV-2 infectious particles per organoid (10⁶ PFU per organoid). Keep some of the organoids for the mock control. Prepare 24 organoids for each condition tested, 12 organoids for qRT-PCR and 12 organoids for immunofluorescence.
29. Infect control or diabetic kidney organoids with 10⁶ PFU per organoid.
 - a. Use wide orifice 200 μ L pipette tips (wo-tips) to place 1 organoid per well of Low-Attachment Surface 96-well plate with 100 μ L per well of the Normoglycemic or Hyperglycemic Medium.

Note: Use always wo-tips to collect or handle organoids.

△ CRITICAL: Keep mock and SARS-CoV-2 infected kidney organoids in separate plates to avoid cross-contamination of the mock controls with virus from infected organoids.

- b. Dilute the virus stock into the desired volume of Complete Infection Medium (Virus Working Solution) to have 10⁶ PFU per 100 μ L (2×10^7 infectious particles/mL).
- c. Remove the media from each well and add 100 μ L of the Virus Working Solution per well of the Low-Attachment Surface 96-well plate containing the organoids. Add the same volume of medium without virus for mock controls.
- d. Incubate the mock or infected organoids at 37°C and 5% CO₂ for 1 h.
- e. After 1 h incubation, remove the supernatant and dispose as infectious waste.
- f. Wash organoids 3 times with 100 μ L of Normoglycemic or Hyperglycemic Medium per well to remove unbound virus. Dispose the supernatants as infectious waste.

⚠ CRITICAL: When performing the washing steps pay attention not to aspirate organoids by accident.

- g. Add 100 μ L per well of fresh Normoglycemic or Hyperglycemic Medium and incubate the mock or SARS-CoV-2 infected organoids at 37°C and 5% CO₂ for 1 day.
30. Collection of infected kidney organoids for further analysis at day 1 post-infection (1 dpi).
 - a. For each condition tested, use a wo-tip to collect 24 organoids in two 1.5 mL Eppendorf tubes (12 organoids per tube).
 - b. Wash the organoids 3 times with 500 μ L of PBS 1 $\times$ per tube to remove unbound virus.
 - c. Remove the supernatant and dispose as infectious waste.
 - d. For viral RNA detection by qRT-PCR, add 500 μ L of Trizol™ to 12 organoids of each condition tested and store the samples at –80°C for further analysis.
 - e. For NP detection by immunofluorescence use 4% PFA solution to fix 12 organoids of each condition tested.
 - i. In the fume hood, add 500 μ L of 4% PFA per Eppendorf tube.
 - ii. Incubate the samples for 12–16 h at 4°C.
 - iii. In the fume hood remove the fixative and wash the fixed organoids 3 times with 500 μ L of PBS 1 $\times$.
 - iv. Keep the fixed samples in PBS 1 $\times$ at 4°C for further analysis.

⏸ Pause point: Fixed organoid specimens are stored at 4°C for further analysis in the following weeks. For longer storage of fixed specimens (up to 6–12 months), we recommend storing them in PBS 1 $\times$ supplemented with 0.02% of Sodium Azide.

Immunofluorescence analysis of kidney organoids upon SARS-CoV-2 infection

⌚ Timing: 4–5 days

This section describes the analysis of the extent of SARS-CoV-2 infection in kidney organoids exposed to Control and Diabetic conditions by immunofluorescence. Kidney organoids are analysed at 1 dpi for the detection of NP in combination with *Lotus Tetragonolobus Lectin* (LTL) to detect proximal tubular-like structures and angiotensin-converting enzyme 2 (ACE2). Immunofluorescence is performed in whole mount (see step 32) or by paraffin-sectioning (see step 33) of the organoids (see [Figure 4](#)).

31. Divide the number of fixed organoid specimens (previously fixed with 4% PFA; see step 30) to perform either whole mount immunofluorescence or immunofluorescence in paraffin sections.

Whole mount immunofluorescence of kidney organoids

⌚ Timing: 4–5 days

32. For whole mount immunofluorescence of kidney organoids proceed as follows (see [Figures 4A](#) and [4B](#)). [Troubleshooting 14](#) and [15](#).

Note: Perform the immunolabelling of at least 3 organoids per experimental condition.

- a. Place 3 organoids per experimental condition in each well of 24-well plate with 400 μ L of Tris-Buffered Saline (TBS) 1 $\times$. Place 1 additional organoid per experimental condition in individual wells of 24-well plate with 400 μ L of 1 $\times$ TBS for secondary antibody controls. This control is necessary to evaluate the potential non-specific staining of secondary antibodies.
- b. Shake the plate in an orbital shaker at 20°C–22°C for 10 min.
- c. Perform the blocking of the organoids.

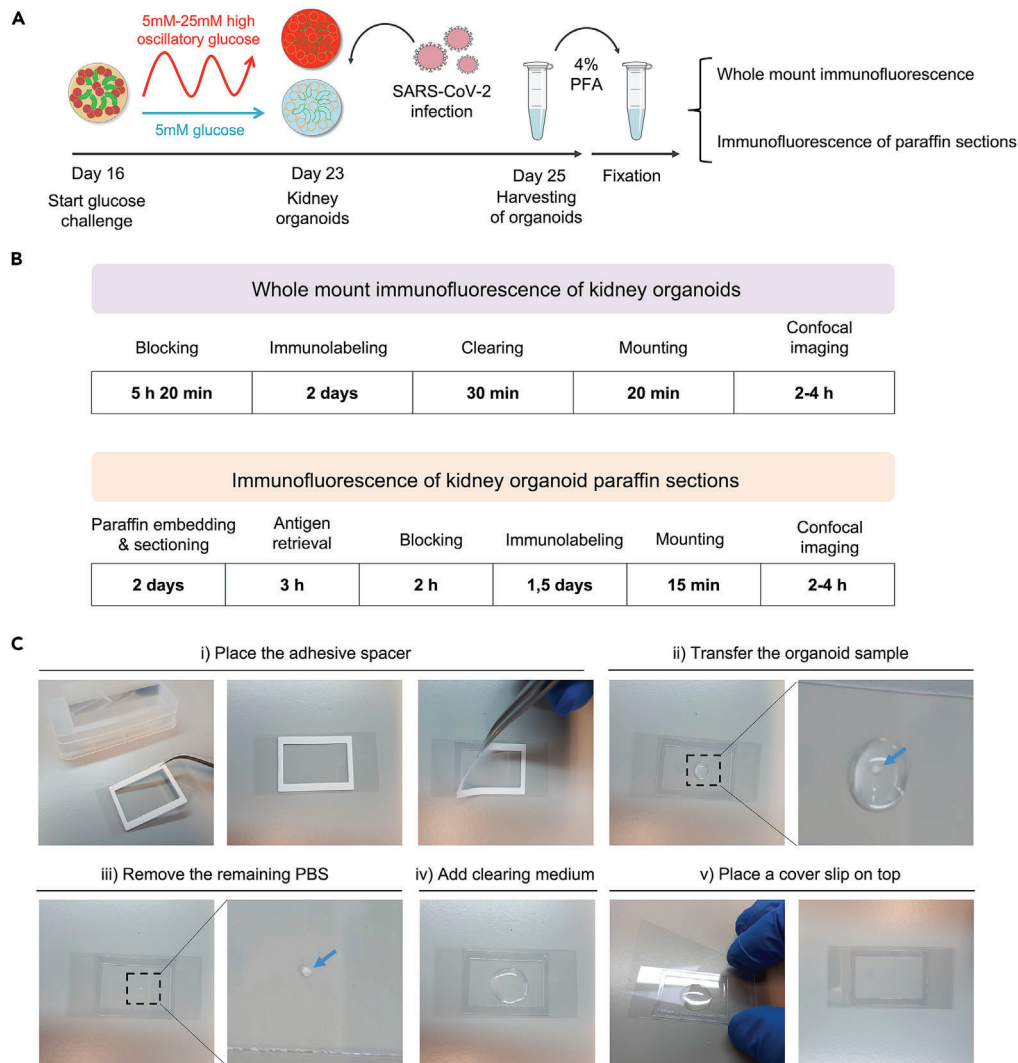


Figure 4. Methodology to analyze kidney organoids by immunofluorescence

(A) Timeline of the procedure to emulate a diabetic-like milieu in kidney organoids, infect them with SARS-CoV-2 virus and then harvest and fix them for performing immunofluorescence analysis.

(B) Overview of the required steps to perform whole mount immunofluorescence or immunofluorescence in paraffin sections of kidney organoids.

(C) Representative photographs to illustrate the procedure for mounting and clearing immunolabeled whole kidney organoid samples for imaging in a confocal microscope.

- i. Remove the TBS 1× from each well of 24-well plate.
- ii. Add 400 µL of TBS 1× supplemented with 1% Triton X-100 and 6% Donkey Serum (6% DS-Blocking Buffer) per well.
- iii. Incubate for 4 h at 20°C–22°C in an orbital shaker.
- d. Perform an additional blocking step with the Streptavidin/Biotin Blocking Kit, composed by a Streptavidin and a Biotin Solution, to block the organoid endogenous biotin. This step is required when biotinylated LTL is used.
 - i. Remove the 6%DS-Blocking Buffer from each well of 24-well plate.
 - ii. Wash 1 time with 400 µL of TBS 1× per well for 15 min at 20°C–22°C in an orbital shaker.
 - iii. Remove the TBS 1× and add 6–8 drops of the Streptavidin Solution to each well to cover the organoids. Incubate for 20 min at 20°C–22°C under constant shaking.

- iv. Wash the organoids once with 400 μ L of TBS 1 $\times$ per well for 15 min at 20°C–22°C in an orbital shaker.
- v. Remove the TBS 1 $\times$ from each well and add 6–8 drops of the Biotin Solution to each well to cover the organoids. Incubate for 20 min at 20°C–22°C under constant shaking.
- vi. Wash the organoids once with 400 μ L of TBS 1 $\times$ per well for 15 min at 20°C–22°C in an orbital shaker.
- vii. Remove the TBS 1 $\times$ from each well and add 400 μ L of TBS 1 $\times$ supplemented with 1% Triton X-100 and 1% Bovine Serum Albumin (BSA) (BSA-1%T-Blocking Buffer)

▮▮ Pause point: At this stage organoids can be stored in BSA-1%T-Blocking Buffer for 12–16 h at 4°C and start the incubation with primary antibodies the following day.

- e. Prepare the necessary primary antibody solution by diluting the correspondent primary antibodies in BSA-1%T-Blocking Buffer (see [materials and equipment](#) section).

Note: Calculate spending 250 μ L of the primary antibody solution per well of the 24-well plate.

- f. Remove the BSA-1%T-Blocking Buffer from each well and cover the organoids with 250 μ L of the primary antibody solution. Incubate for 1 h at 20°C–22°C under constant shaking, then 12–16 h at 4°C and finally at 20°C–22°C for an additional 1 h, under shaking conditions.

⚠ CRITICAL: Remember to add 1 organoid per condition as the secondary antibody control. Leave the secondary antibody control organoid in BSA-1%T-Blocking Buffer without the addition of primary antibodies.

Optional: Primary antibody incubation at 4°C can be extended up to 24–48 h.

- g. After primary antibody incubation, wash organoids 3 times with 400 μ L of BSA-1%T-Blocking Buffer per well for 20 min at 20°C–22°C under shaking conditions.
- h. Prepare the necessary secondary antibody solution by diluting the correspondent secondary antibodies in BSA-1%T-Blocking Buffer (see [materials and equipment](#) section).

Note: Calculate spending 250 μ L of the secondary antibody solution per well of the 24-well plate. Remember to include the secondary antibody control organoid incubating it with the secondary antibody solution.

- i. Centrifuge the secondary antibody solution at 1,200 $\times g$, take the supernatant and discard any pellet of precipitates that might have been formed.

⚠ CRITICAL: The potential presence of precipitates in the secondary antibody solution could interfere with the imaging of organoids by confocal microscopy.

- j. Remove the BSA-1%T-Blocking Buffer from each well and cover the organoids with 250 μ L of the secondary antibody solution per well. Incubate for 4 h at 20°C–22°C in an orbital shaker in darkness. Maintain the darkness conditions until the end of the protocol.

⚠ CRITICAL: Prolonged incubation with secondary antibody solution for more than 4 h might cause unspecific antibody adhesion to the organoids.

- k. After secondary antibody incubation, wash the organoids 3 times with 400 μ L of TBS 1 $\times$ per well for 20 min at 20°C–22°C under shaking conditions.
- l. Counterstain the nuclei with DAPI.

- i. Remove the TBS 1× from each well and cover the organoids with 250 µL of the DAPI working solution (dilute the DAPI stock at 1:5000 ratio in TBS 1×).
- ii. Incubate for 12–16 h at 4°C.
- iii. Remove the DAPI solution and add 400 µL of PBS 1× to each well. Keep the immunolabelled organoids at 4°C in the darkness until mounting them for imaging.

Note: Avoid storage of immunolabelled organoids longer than 24–48 h.

- m. Perform clearing and mounting of the samples for confocal imaging (See [Figure 4C](#)).
 - i. Take 1 rectangular cover slip per sample condition and adhere a double-sided sticky iSpacer®.
 - ii. Using a wo-tip, transfer 1 immunolabelled organoid, trying to collect the minimum amount of PBS 1×, onto the cover slip. Use a tip to remove as much as possible any residual PBS 1× solution.

Note: 3 organoids per experimental condition can be cleared and mounted in the same rectangular cover slip.

- iii. Add a drop of 100 µL of RapiClear® 1.47 clearing reagent to completely immerse the organoid in the clearing solution. Avoid introducing bubbles.
- iv. Incubate for 20 min at 20°C–22°C until the organoid becomes transparent.
- v. Place another rectangular cover slip on top of the cleared organoid and check that the clearing solution spreads and cover the entire surface. The double-sticky iSpacer® allows adhesion of both cover slips.
- vi. Store the mounted organoid samples at 4°C in the darkness until imaging.

Immunofluorescence of kidney organoid paraffin sections

⌚ **Timing:** 4 days

33. For immunofluorescence of kidney organoid paraffin sections proceed as follows (see [Figures 4A and 4B](#)). [Troubleshooting 16](#).
 - a. Prepare organoids for paraffin-embedding.
 - i. Heat the 0.8% low melting agarose solution at 60°C in a water bath until having a liquid agarose solution. Maintain the agarose solution warm at 37°C.
 - ii. Place a layer of 100 µL of 0.8% low melting agarose solution into the bottom of a plastic cryomold. Allow to cool down for 5 min.
 - iii. Using a wo-tip, immediately re-suspend the fixed kidney organoids samples (from step 31) in 150 µL of warm 0.8% low melting agarose (37°C) and transfer the organoid–agarose suspension to the agarose-coated plastic cryomold.

Note: Place at least 3 fixed kidney organoid specimens per cryomold.

⚠ CRITICAL: Ensure that organoids are surrounded by agarose without introducing air bubbles. Place the organoids close to each other.

- iv. Place the prepared molds on ice until the agarose is solidified.
- v. Remove the agarose block containing the organoids from the mold and place it in 1 well of 12-well plate with 1 mL of cold PBS 1×.

⏸ Pause point: The agarose blocks with organoids can be stored at 4°C, up to 1 week, for further paraffin embedding. We recommend to store agarose blocks in PBS 1× supplemented with 0.02% of Sodium Azide.

- b. Make paraffin blocks.
 - i. Using a spatula, transfer the agarose blocks prepared in step 33a into embedding cassettes.
 - ii. Proceed to standard dehydration in an automatic tissue processor (Tissue-Tek® VIP® 6, Sakura or equivalent) following the standard procedure.
 - iii. Embed in paraffin at 58°C using metallic molds to obtain paraffin blocks in a paraffin station (Tissue-Tek TEC, Sakura or equivalent).

▮▮ Pause point: The paraffin blocks can be stored at 20°C–22°C.

- c. Section the paraffin blocks.
 - i. Cool down the paraffin blocks on a cold plate (Tissue-Tek, Sakura or equivalent).
 - ii. Prepare organoid sections of 3 µm-thickness using a rotary microtome (Leica RM2255 or equivalent).

△ CRITICAL: Since organoids are quite small and difficult to distinguish within the paraffin block, do not approach the sample by cutting thicker sections to avoid losing the sample.

- iii. Guide the ribbon of paraffin sections into the water bath (Leica HI1210 or equivalent) at 40°C to help unfolding of the sections on the water surface.
- iv. Collect the paraffin sections using charged glass slides. Prepare 20 slides with 2 sections per slide.

△ CRITICAL: Properly label each slide.

Note: We recommend performing serial sectioning.

- v. Dry the slides in an oven at 60°C for 12–16 h.

▮▮ Pause point: The slides can be stored at 20°C–22°C in a dry place for 6–12 months.

- d. Select the slides for immunofluorescence analysis.
 - i. Perform Hematoxylin and Eosin (H&E) staining in slides number 5, 10, 15 (from a total of 20 prepared slides per paraffin block) following standard procedures.
 - ii. Observe the H&E-stained slides under the microscope to identify the slides that contain organoid sections.

Note: For example, if organoid sections are visible by H&E staining in slides 5 and 10 but not in slide 15, this indicates that organoids sections are majorly present from slide 5 to slide 10.

- iii. Select at least 2 slides per sample to perform immunofluorescence analysis of a given combination of primary antibodies. Take an additional slide for performing the secondary antibody control.
- e. Deparaffinize and re-hydrate the selected slides.
 - i. Use an automated tissue processor following the standard procedure.
- f. Perform antigen retrieval.
 - i. Immerse the slides in citrate buffer (pH 6).
 - ii. Autoclave the immersed slides at 95°C for 20 min.
 - iii. Cool down the slides to 20°C–22°C.

▮▮ Pause point: After antigen retrieval the slides can be stored at 4°C in TBS 1 × for 24 h before continuing with the next step.

- g. Perform the blocking of the samples.
 - i. Wash the slides with 0.5–1 mL TBS 1× per slide.
 - ii. Use 0.5–1 mL of TBS 1× containing 1% Triton X-100 and 3% Donkey Serum (3%DS-Blocking Buffer) to cover the organoid sections in each slide.
 - iii. Incubate the samples for 1 h at 20°C–22°C.

Note: Use a box for histology slides to make a humidified chamber and horizontally place the slides inside to perform the blocking and the subsequent immunolabelling steps.

- h. Perform an additional blocking step with the Streptavidin/Biotin Blocking Kit as detailed in step 32d. This step is required when biotinylated LTL is used. After that, cover the organoid sections in each slide with 0.5–1 mL of TBS 1× containing 0.5% Triton X-100 and 1% BSA (BSA-0.5%T-Blocking Buffer).

Note: Shaking is not required.

- i. Prepare the necessary primary antibody solution by diluting the corresponding primary antibodies in BSA-0.5%T-Blocking Buffer (see [materials and equipment](#) section).

Note: Calculate spending 200 µL of the primary antibody solution per slide.

- j. Remove the BSA-0.5%T-Blocking Buffer from the slides and use a hydrophobic barrier pen to draw a circle around organoid sections in each slide.
- k. Cover organoid sections in each slide with 200 µL of the primary antibody solution. Incubate the slides for 12–16 h at 4°C.

△ CRITICAL: Remember to use 1 slide for the secondary antibody control. Leave the secondary antibody control slide in BSA-0.5%T-Blocking Buffer during primary antibody incubation.

△ CRITICAL: Use a box for histology slides to make a humidified chamber and horizontally place the slides inside. Ensure to place the box containing the slides in a quiet place in the refrigerator to avoid any movement or vibration.

- l. After primary antibody incubation, wash the slides 3 times with 0.5–1 mL of BSA-0.5%T-Blocking Buffer per slide for 5 min at 20°C–22°C.
- m. Prepare the necessary secondary antibody solution by diluting the corresponding secondary antibodies in BSA-0.5%T-Blocking Buffer (see [materials and equipment](#) section).

Note: Calculate spending 200 µL of the secondary antibody solution per slide.

- n. Cover organoid sections in each slide with 200 µL of the secondary antibody solution. Incubate the slides for 2 h at 20°C–22°C in darkness.

△ CRITICAL: Ensure to place the box containing the slides in a quiet place to avoid any movement or vibration.

- o. After secondary antibody incubation, wash the slides 3 times with 0.5–1 mL of TBS 1× per slide for 5 min at 20°C–22°C.
- p. Counterstain the nuclei with DAPI for 30 min at 20°C–22°C (see step 32l).
- q. Mount the slides for imaging.
 - i. Remove the DAPI solution from the slides.
 - ii. Deposit 3–4 drops of Fluoromount-G mounting medium on top of each slide.

⚠ CRITICAL: Avoid bubble formation.

- iii. Immediately place rectangular coverslip on top of the slide. Carefully remove the mounting media that may come out from the slide using absorbent paper.
- iv. Seal the borders of the slide with nail polish.

▮▮ Pause point: The mounted slides can be stored at 4°C in darkness for 1–2 weeks without substantial decrease in fluorescence signal intensity.

34. Acquire images on a confocal microscope. Use the Fiji ImageJ2 version 2.3.0 software for image processing and generation of TIFF files.

Note: Zeiss LSM780 or LSM 880-Airyscan Elyra confocal microscopes were used although other confocal microscopes can be used to obtain similar outcomes. Commonly used acquisition settings would be: scan mode= frame; frame size= 1024 × 1024; line average= 2; bidirectional scanning; speed= 7. A 10× dry objective (Plan-Apochromat 10×/0.3 M27) or a 25× multi-immersion objective (Plan-Apochromat 25×/0.8 Imm Corr DIC M27) can be used to acquire the whole organoid specimen or organoid section by activating the tile scan mode to indicate the borders of the organoid in the x/y direction. The z-stack mode is also activated to define the upper and lower limits of the sample in the z direction. A 40× oil immersion objective (Plan-Apochromat 40×/1.3 Oil DIC M27) can be used for imaging high magnification details of the sample.

EXPECTED OUTCOMES

Following the procedures detailed here we routinely produce SARS-CoV-2 virus stocks and assess virus stock titers in 95% of plates from which virus production are initiated, obtaining virus stock titers ranging from 10^6 and 10^7 PFU/mL.

Additionally we expect the efficient generation of kidney organoids from hPSC in 90% of plates from which differentiations are initiated. Following our methodology kidney organoids develop in free floating conditions using V-bottom 96-well plates in which 1 single organoid forms in each well.

Successful generation of kidney organoids is characterized by the formation of round translucent epithelial structures, so-called RV, visible under the microscope on day 11 of differentiation (Figure 3H), and then progress into nephron-like structures until day 16 of differentiation (Figure 3J). Our procedure leads to the generation of kidney organoids containing segmented nephron-like structures as well as stromal-like cells, endothelial-like cells, among others.^{1–4}

Recently, we established a high oscillatory glucose regime that leads to early hallmarks of diabetic kidney disease during development and investigated the impact of a diabetic-like milieu on early stages of SARS-CoV-2 infection in hPSC-derived kidney organoids.¹ Our procedure is highlighted in Figures 5A and 5B. Here, we provide representative confocal images of control and diabetic organoids showing that ACE2 expressing cells (ACE2⁺) are mainly detected within LTL positive proximal tubule-like structures (LTL⁺) (Figures 5C and 5D).¹

Here we also detail methods for the detection of SARS-CoV-2 infection in control or diabetic kidney organoids taking advantage of whole mount immunofluorescence and immunofluorescence in paraffin sections. In our recent manuscript both immunolabelling techniques have been optimized and validated for the efficient detection of ACE2⁺ and NP expressing cells (NP⁺) along with different renal markers, such as LTL for proximal tubule structures, WT1 for podocyte-like cells, or CD31 for endothelial-like cells.¹

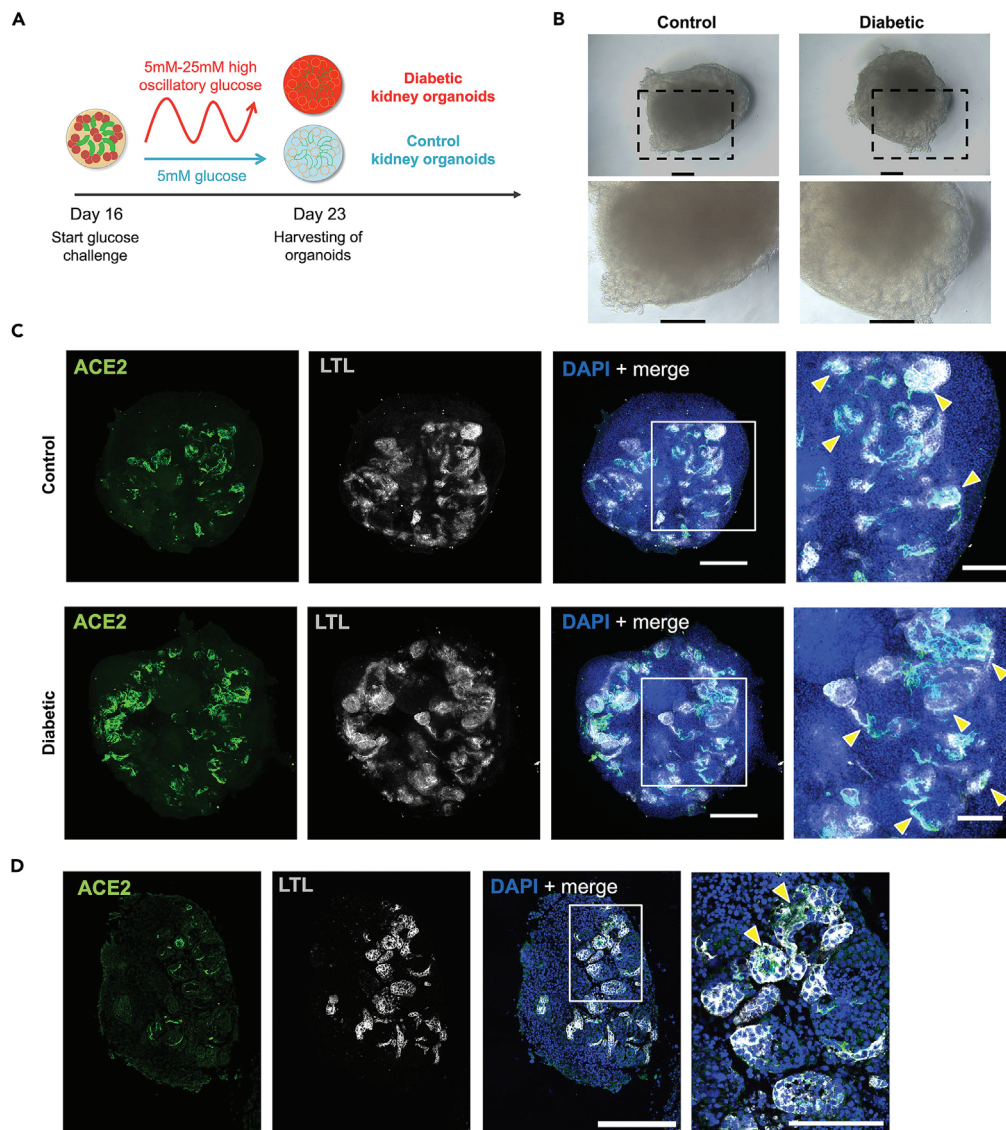


Figure 5. Immunofluorescence analysis of kidney organoids after exposure to diabetogenic-like culture conditions

(A) Schematic overview of the procedure to induce control or diabetic kidney organoids.

(B) Representative bright field images of control or diabetic kidney organoids. Scale bars, 100 μ m.

(C) Representative confocal images of control or diabetic kidney organoids analyzed by whole mount immunofluorescence for the detection of ACE2 (green), LTL (gray) and DAPI (blue). Scale bars, 250 μ m, 100 μ m (high magnification views).

(D) Example of representative confocal images that show the detection of ACE2 (green), LTL (gray) and DAPI (blue) by immunofluorescence in an organoid paraffin section. Scale bars, 250 μ m, 100 μ m (high magnification view). Yellow arrowheads in (C-D) indicate examples of LTL positive proximal tubule-like structures with ACE2 expressing cells.

Here, we provide the step-by-step procedure for the detection of viral NP together with ACE2 and LTL in mock or SARS-CoV-2 infected kidney organoids under control or diabetic conditions. In [Figure 6](#) we provide representative confocal images of immunolabeled whole mount organoids ([Figure 6A](#)) or organoid paraffin sections ([Figure 6B](#)) showing that NP⁺ are mainly detected within LTL⁺ in infected organoids.

Overall, the methodology described herein enables side-by-side comparison of SARS-CoV-2 infectivity between control and diabetic kidney organoids. Our read outs allow for the examination of viral

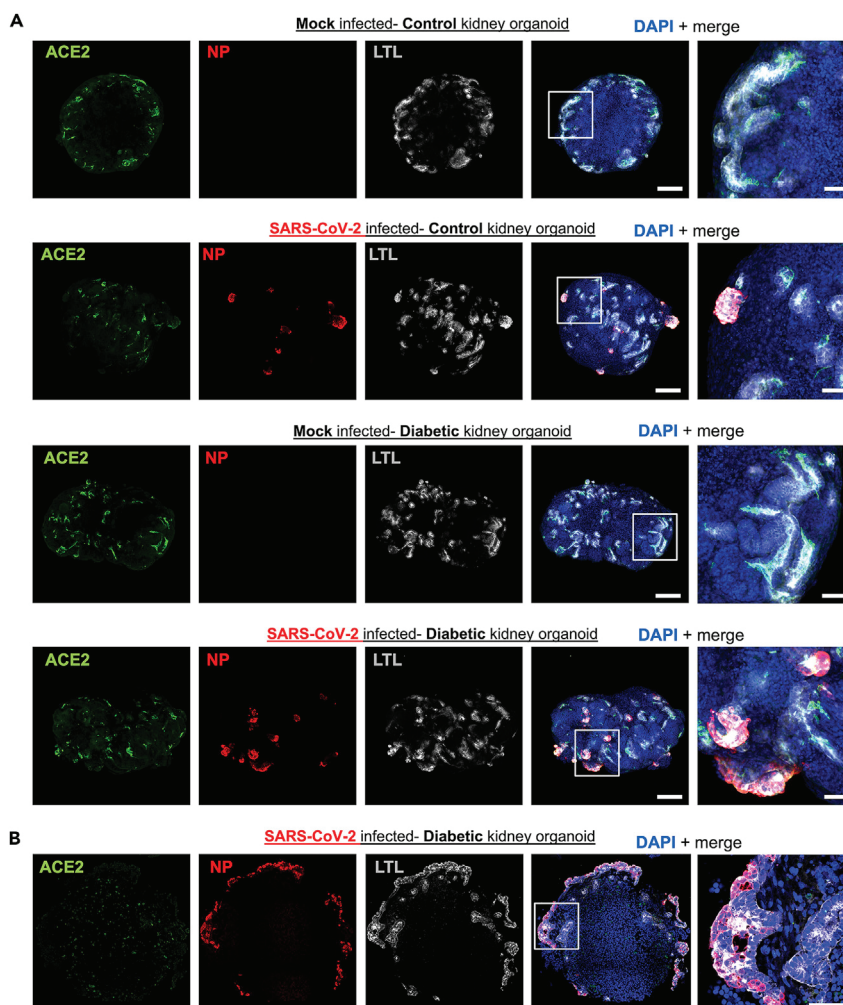


Figure 6. Immunofluorescence analysis of control and diabetic kidney organoids upon SARS-CoV-2 infection

(A) Representative confocal images of mock or SARS-CoV-2 infected kidney organoids at 1 dpi for the detection of ACE2 (green), NP (red), LTL (gray) and DAPI (blue) by whole mount immunofluorescence. Scale bars, 200 μ m, 50 μ m (high magnification views).

(B) Example of representative confocal images that show the detection of ACE2 (green), NP (red), LTL (gray) and DAPI (blue) by immunofluorescence in an organoid paraffin section. Scale bars, 200 μ m, 50 μ m (high magnification view).

NP expression concomitantly with ACE2 and LTL. Further analysis using markers of major kidney compartments can be found in our recent manuscript.¹

LIMITATIONS

The protocol described herein has been successfully used to study the impact of a diabetogenic-like milieu on early stages of SARS-CoV-2 infection in hPSC-derived kidney organoids.¹ However, we are aware that *in vitro* models as organoids do not truly recapitulate the full complexity of the human organ. For instance, it should be noted that the procedure described here to generate kidney organoids does not allow for the generation of fully developed vascular networks neither immune cells nor a proper collecting duct system. Nevertheless, our renal differentiation procedure consistently gives rise to kidney organoids containing ACE2⁺ within tubular-like structures,¹ as well as contain other renal cell populations (i.e., podocyte-like cells, tubule-like cells, endothelial-like cells, among others),^{1–4} recapitulating in part the multicellular complexity of the native organ and thus providing a valuable *in vitro* model to study SARS-CoV-2 infection in the human context.

In our recent study we have made use of 2 different hPSC lines for the derivation of kidney organoids, including male human embryonic stem cells (ES[4] cell line) and female cord blood iPSCs (CBiPS1sv-4F-40 cell line), confirming infection with SARS-CoV-2 in both genetic backgrounds under non-diabetic and diabetic-like conditions.¹ It is well known that to model infection successfully it is crucial to ensure the accessibility of the different cell types in the organoid model compared to the native organ.⁵ In this regard, it is important to highlight that ACE2⁺ in kidney organoids are spontaneously exposed and accessible to *in vitro* interventions as SARS-CoV-2 infection. Another important aspect in this regard is that our procedure does not require extracellular matrix (ECM)-related matrices (i.e., Matrigel) for organoid culture and thus viral accessibility is equal between biological replicates. However, as the organoid model system defined here does not recapitulate important features as proper vascularization or immune system, there remains a lack of understanding on complex cell-to-cell interactions and crosstalk upon infection. Further aspects to be studied in the future include exposure time by increasing organoid lifespan and sample analysis upon longer periods post-infection.

TROUBLESHOOTING

Problem 1

Low cell viability after thawing a cryovial of hPSC on VTN-N-coated plates in Complete E8 Medium. 24 h after thawing hPSC have not attached and most of them appear as floating dead cells in the culture plate ([before you begin](#), step 10; see [Figure 1B](#)).

Potential solution

Ensure that hPSC colonies are at 80% confluency and have the correct morphology (without spontaneous differentiation) before freezing. When freezing hPSC, ensure to gently dissociate hPSC colonies in cell clusters avoiding single cell dissociation and use E8 medium supplemented with 10% DMSO as freezing media. For successful cryopreservation of cells, use a freezing container (i.e., Nalgene® Mr Frosty) to store the cryovials at -80°C . This type of containers ensures a controlled slowdown of the cryovials temperature at $1^{\circ}\text{C}/\text{min}$ cooling rate until reaching -80°C . Keep the cryovials at -80°C for 24 h after which immediately transfer the frozen cryovials to the liquid nitrogen tank. It is also important to thaw quickly the cryovial at 37°C and immediately wash cells to remove the DMSO by adding 10 mL of Complete E8 Medium in a 15 mL falcon tube, centrifuging the cell suspension (5 min, $300 \times g$) and gently re-suspending the cell pellet in fresh Complete E8 Medium. Do not use rock inhibitor.

Problem 2

Low cell viability after passaging. High cell mortality and too low cell attachment is observed after performing the passage ([before you begin](#), step 13; see [Figure 1C](#)).

Potential solution

Ensure to passage hPSC colonies with the right confluency (80%) and morphology, without spontaneous differentiation. Adjust the timing of exposure to EDTA. Too long EDTA exposure may result in too small cell clusters or single cells, reducing viability. Too short EDTA exposure may result in insufficient cell detachment and exposure of cells to high mechanical stress when trying to detach them, thus reducing viability. After EDTA exposure, use 1 mL of Complete E8 Medium to flush the well and detach the hPSC colonies. They should come out easily. Use another fresh mL of E8 medium to flush again the well and collect the remaining cells. Avoid excessive up and down pipetting of the hPSC clumps suspension. Do not use rock inhibitor to passage the hPSC. Revise that VTN-N coatings have been properly prepared. Avoid using prepared VTN-N coatings that have been stored for more than a week. Remember to routinely check the expiration date and batch of VTN-N and Complete E8 Medium.

Problem 3

hPSC colonies do not have defined edges, or cells appear less compact within the colonies, or large differentiated cells appear in the culture within or surrounding the hPSC colonies ([before you begin](#), step 13; see [Figure 1D](#)).

Potential solution

Ensure to use freshly prepared VTN-N coatings, Complete E8 Medium and EDTA solution. Ensure to make periodic medium changes to hPSC cultures as recommended. Reduce hPSC colonies density by increasing the splitting dilution ratio. Do not use rock inhibitor for passaging hPSC.

Problem 4

When plates are revealed with crystal violet staining, plaques are found to be grouped around edges of wells or uneven distributed within wells (step 12 of [step-by-step method details](#)).

Potential solution

Virus inoculum was not well distributed during incubation of virus dilutions for 1 h. Make sure to distribute the virus inoculum across the wells by gently moving the plates from front to back and side to side every 15 min of incubation to allow homogeneous distribution of virus.

Problem 5

A large area of missing cells or a large plaque-like spot is observed (step 12 of [step-by-step method details](#)).

Potential solution

This could be caused by scratching them when removing the agarose with the spoon. To avoid this, be careful not to touch monolayers with the flat spoon to prevent harming the cell monolayers.

Problem 6

The number of counted plaques is lower than 5 in all virus dilutions, or the number of counted plaques is bigger than 100 in all virus dilutions (step 13 of [step-by-step method details](#)).

Potential solution

If number of plaques is lower than 5 at all dilutions, this means that virus titer may be less than 1×10^2 PFU/mL. Discard the virus stock and consider making new virus stocks to increase the virus titer. If number of plaques is bigger than 100 at all dilutions, this means that virus titer may be higher than 1×10^7 PFU/mL. In this case, repeat the plaque assay performing further serial dilutions: 10^{-6} , 10^{-7} and 10^{-8} .

Problem 7

Low virus titers are obtained (step 13 of [step-by-step method details](#)).

Potential solution

In our hands, most SARS-CoV-2 virus stock titers are between 10^6 to 10^7 PFU/mL. With this as reference, some methodological points can be considered to achieve optimal virus titers. (a) Routinely check morphology of Vero-E6 Cells and change to low passage Vero-E6 Cell stocks if any change is appreciated or infectivity has decreased. (b) For proper virus production, infect Vero-E6 Cells at low MOI per cell, usually 0.01. (c) To guarantee that virus supernatants are collected at the optimal time (when virus production peaks), prepare additional flasks to collect the virus supernatants at different time points and check the best titer by the plaque assay.

Problem 8

24 h after seeding the hPSC in the 24-well plate for differentiation, hPSC appear all together in the middle of the well or uneven distributed across the well, or heterogeneity on cell density is observed among different wells (step 21 of [step-by-step method details](#)).

Potential solution

When plating the hPSC into the 24-well plate, dispense 0.5 mL of the cell suspension per well of the 24-well plate using a p1000-micropipette or a 5 mL-pipette while making sure to homogenize the

suspension of cell clumps regularly by gently moving the falcon tube. After plating the cells, manually shake the 24-well plate to allow homogeneous distribution of the cell clumps across the wells, repeat after 30 min. Then, keep the plates in a quite incubator, avoiding any vibration or movement (i.e., opening-closing the incubator) for at least 2 h.

Problem 9

Cells massively die after CHIR addition (from day 0 to day 3 of renal differentiation), (step 22 of [step-by-step method details](#)).

Potential solution

It is important to take in mind that hPSC seeding density and colony distribution are crucial for achieving efficient renal differentiation and formation of a compact cell monolayer by day 4 of differentiation. If there is excessive cell death upon CHIR stimulation, this might be due to inadequate cell density or inadequate colonies morphology at day 0 of the protocol. For optimal colony density and morphology on day 0 see [Figure 3B](#). If colonies are too small after 24–48 h of plating, replace the media with fresh Complete E8 Medium and wait additional 24 h before starting the differentiation. It is recommendable to optimize the initial cell seeding density for each hPSC line. If above reasons have been excluded, revise that CHIR stocks have been correctly prepared.

Problem 10

On day 4 of differentiation cells have not formed a confluent and compact cell monolayer as expected at this stage. Instead, areas of loose cells or empty areas are observed in the cultures (see [Figure 3D](#), step 23 of [step-by-step method details](#)).

Potential solution

This could be due to improper hPSC density and colony distribution when starting the differentiation at day 0 of the kidney organoid generation protocol. If this is the case, repeat the experiment using 2 different initial hPSC seeding densities to ensure that 48 h after hPSC seeding, the required confluency and colony distribution is optimal to start the differentiation process. Also, from day 0 to day 4 of the differentiation protocol, take special care when performing media changes since differentiated cells tend to easily detach from the plate. Thus, dispense media slowly to avoid producing excessive pressure against the cells that would damage the cell monolayer and impair the formation of a compact cell monolayer by day 4 of differentiation.

Problem 11

On day 6 of the kidney organoid generation protocol cell spheroids do not have a compact morphology with defined edges. Instead, cell spheroids appear disaggregated (see [Figure 3F](#), step 24e of [step-by-step method details](#)).

Potential solution

Repeat the experiment and make sure to check the following methodological points. On day 4 of differentiation: (a) when dissociating the cell monolayers into single cells, avoid excessive exposure of the cell monolayer to TrypLE™ Express solution, and completely remove the TrypLE™ Express solution before dissociating the cell monolayer by flushing the cells with media. Do not preform excessive up and down pipetting of the cell suspension since this may harm the cells; (b) after dissociation collect the cell suspension in kidney organoid differentiation medium containing FGF9 and heparin. Do not use medium without FGF9 and heparin supplementation. While counting the cells, keep the cell suspension in the falcon tube at 37°C to preserve cell viability; (c) after cell counting and plating in V-bottom 96-well plates, check that the centrifuge is properly equilibrated when V-bottom 96-well plates are centrifuged to force the aggregation of day 4-differentiated cells into spheroids. After centrifuging the plates, check that a pellet of cells is properly formed at the bottom of each well of the V-bottom 96-well plate. After 48 h of incubation (day 6), each cell pellet should result in the formation of a spheroid.

Problem 12

On day 11 of the kidney organoid generation protocol RV structures are not visible within organoids under the optical microscope (see [Figure 3I](#), step 24h of [step-by-step method details](#)).

Potential solution

This is due to failed renal differentiation. Repeat the experiment. Check that the initial hPSC density is the optimal for efficient kidney organoid generation (see [Figure 3B](#)) and perform media changes regularly as explained in each step of the protocol. Also, revise that the stocks of factors and reagents for medium preparation and basal medium for kidney organoid generation have not expired. Avoid repeated freezing and thawing cycles of the aliquoted stocks and avoid using reconstituted stocks of CHIR, FGF9, Activin A, and heparin for more than 6 months.

Problem 13

On day 16 of kidney organoid generation nephron-like structures are not observed within organoids under the optical microscope (see [Figure 3K](#), step 25 of [step-by-step method details](#)).

Potential solution

This is due to failed or inefficient renal differentiation. It is possible that the yield of kidney differentiation was low, leading to the formation of other cell types that overgrow within the organoid, thus impairing the development of the few renal structures that could have formed. Repeat the experiment to improve the yield of renal differentiation by following specific recommendations detailed in the protocol herein (i.e., proper initial cell density and colony distribution by day 0, formation of a proper homogeneous and compact cell monolayer by day 4, perform media changes regularly as explained in each step of the protocol, among others). Also, revise that the stocks of factors and reagents for medium preparation and basal medium for kidney organoid generation have not expired. Avoid repeated freezing and thawing cycles of the aliquoted stocks and avoid using reconstituted stocks of CHIR, FGF9, Activin A, and heparin for more than 6 months.

Problem 14

After performing whole mount immunofluorescence of kidney organoids, the organoid samples used for secondary antibodies control display a high background signal (step 32 of [step-by-step method details](#)).

Potential solution

The secondary antibodies detailed in the protocol herein have been extensively tested and successfully validated for whole mount immunolabelling of kidney organoids with reproducible results. However, make sure to avoid incubating samples with secondary antibodies solution for more than 4 h since this may promote unspecific antibodies binding and cause small antibody precipitates. Also, after secondary antibodies incubation you can perform additional washing steps with increased duration time (i.e., 4 times for 30 min each). Ensure to maintain samples under shaking while performing the antibodies incubation and washing steps.

Problem 15

After performing whole mount immunofluorescence of kidney organoids weak signal of immunolabelled proteins is observed across the organoid sample (especially in the middle of the organoid) by confocal microscopy (step 32 of [step-by-step method details](#)).

Potential solution

This could be caused by inefficient antibodies penetration within the organoid samples. The whole mount immunofluorescence protocol detailed herein has been optimized for the efficient immunolabelling of kidney organoids. Ensure to maintain samples under shaking while performing the antibodies incubation and washing steps. Primary antibodies incubation step can be extended up to 48 h.

Problem 16

After performing immunofluorescence of kidney organoid paraffin sections, uneven immunolabelling of organoid sections is observed by confocal microscopy (step 33 of [step-by-step method details](#)).

Potential solution

This could be caused by heterogeneous exposure of the antibody solution in the sample. Ensure that there has not been any problem during de-paraffinization and re-hydration of organoid paraffin sections. Ensure that organoid sections do not dry during any of the immunolabelling steps, especially during primary antibodies incubation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nuria Montserrat (nmontserrat@ibecbarcelona.eu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.M.; Methodology, A.M., S.B., J.M.P., N.M.; Investigation, E.G., D.M-R., M.L.S., V.M., P.P., A.M., C.T., M.G., G.J., A.H.; Writing – Original Draft, E.G., N.M.; Writing –

Review & Editing, E.G., M.L.S., V.M., C.T., A.M., S.B., J.M.P., N.M.; Funding Acquisition, A.M., S.B., J.M.P., N.M.; Resources, A.M., S.B., J.M.P., N.M.

DECLARATION OF INTERESTS

A patent has been submitted to use human organoids to study SARS-CoV-2 infections and possibly develop new therapies. J.M.P. is a shareholder of Apeiron Biologics, which is developing ACE2 decoys for COVID-19 therapy.

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